EVALUVATION OF ANALGESIC, ANTI-INFLAMMATORY AND CYTOTOXIC STUDIES OF THE METHANOLIC EXTRACT OF *SPHENODESME PANICULATA* (Clarke) IN EXPERIMENTAL ANIMALS

Dissertation Submitted to

The Tamil Nadu Dr. M.G. R.Medical University, Chennai,

in partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY
(Pharmacology)
MARCH 2016

Submitted by

Reg No: 261425809

Department Of Pharmacology

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPATTI ROAD,

COIMBATORE - 641 048

Prof. DR. A. RAJASEKARAN, M. Pharm., Ph.D.,

Principal,
KMCH College of Pharmacy,
Kovai Estate, Kalapatti Road,
Coimbatore - 641 048,
Tamil Nadu.

CERTIFICATE

This is to certify that the dissertation work entitled **"Evaluvation of analgesic, anti-inflammatory and cytotoxic studies of the methanolic extract of *sphenodesme paniculata* (Clarke) in experimental animals"**submitted by **Akbarali UK,**i s a bonafide work carried out by the candidate under the guidance of **M. Senthilkumar, M Pharm., (Ph.D.,) Assistant Professor** and submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of **MASTER of PHARMACY** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, during the academic year march **2015-2016**.

DATE: **Prof. DR. A. RAJASEKARAN, M. Pharm., Ph. D.,**

PRINCIPAL

M.SENTHIL KUMAR, M. Pharm., (Ph.D).,

Assistant Professor ,
KMCH College of Pharmacy,
Kovai Estate, Kalapatti Road,
Coimbatore - 641 048,
Tamil Nadu.

CERTIFICATE

This is to certify that the dissertation work entitled **"Evaluvation of analgesic, anti-inflammatory and cytotoxic effect of the methanolic extract of *Sphenodesme paniculata*(clarke) in experimental animals"** by **Akbarali UK** is a bonafide work carried out by the candidate under my guidance and submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of **MASTER of PHARMACY** at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year **2015-2016**.

Date

M. SENTHILKUMAR, M Pharm, (Ph.D).,
Assistant Professor,
Department of Pharmacognosy

DECLARATION

I do hereby declare that the dissertation work entitled **"Evaluvation ofanalgesic, anti-inflammatory and cytotoxic studies of the methanolic extract of *Sphenodesme paniculata*(Clarke) in experimental animals"** by **Akbarali UK.** submitted to The Tamil Nadu Dr. M. G. R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy (Pharmacology)** was done by me under the guidance of **M. SENTHILKUMAR, M Pharm, (Ph.D)., Assistant Professor,** at the Department of Pharmacognosy, KMCH College of Pharmacy, Coimbatore, during the academic year **2015-2016**.

Date : **Akbarali UK**

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Evaluvation of analgesic, anti-inflammatory and cytotoxic studies of the methanolic extract of *Sphenodesme paniculata* (Clarke) in experimental animals"** submitted by **Akbarali UK (Reg No: 261425809)** to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy in Pharmacology** is a bonafide work carried out by the candidate at the Department of Pharmacology, KMCH College of Pharmacy, Coimbatore, Tamil Nadu, and was evaluated by us during the academic year **2015-2016**.

Examination Centre: KMCH College of Pharmacy, Coimbatore.

Date

Internal Examiner **External Examiner**

Convener of Examinations

ACKNOWLEDGEMENT

My dissertation entitled **"Evaluvation of analgesics and anti inflamatory activities of the extract of *Sphenodesme paniculata* (Clarke)** *would not have been a feasible one without the grace of God almighty who gave me moral till the completion of my project.*

I extend my thanks to our respected chairman ***Dr.NALLA.G.PALANISWAMI, MD, AB(USA)*** *and respected trustee madam* ***Dr.THAVAMANI D. PALANISWAMI, MD, AB (USA),*** *Kovai Medical Center Research and Education Trust, Coimbatore for the facilities provided by them to carry out this project in a nice manner.*

I extend my gratitude to ***DR. A. RAJASEKARAN, M.Pharm., Ph.D.,*** *Principal, KMCH College of Pharmacy, Coimbatore, for his constant encouragement, support and facilities provided.*

*First and foremost it gives me great pleasure to record my deep sense of gratitude and indebtedness to my esteemed guide.***"M. SENTHILKUMAR, M. Pharm, PhD., Assistant Professor**"**,***Department of Pharmacognosy of KMCH College of Pharmacy, for his constant insight, guidance, countless serenity, encouragement and pain taking efforts in my project work . I am indebted to his kindness and never failing co-operation.*

I owe my heartfelt thanks to my esteemed and beloved staffs ***DR. K.T. Manisenthil Kumar, M.Pharm., Ph.D., Mr. G. AriharaSivakumar, M.Pharm,Dr. N. Adhirajan, M.Pharm., Ph.D.,Mr. C. Sundaramoorthy, M.Pharm.,*** *for their sensible help and suggestions.*

I dedicate myself before the unfailing presence of ***GOD*** *and constant love and encouragement given to me by my beloved Father****,*** *my mother, my brothersand my remaining family members who deserve the credit of success in whatever work I did.*

.

A journey is easier when you travel together; independence is certainly more valuable than dependence. So my sincere thanks and differential regards to my friends ***Amal Tom Alex****,* ***Shyaleen, Bhavya, Sneha, Dini, Anju, Thafla, Rekha, G.Krishnareddy, AaronMathew, Dani, DivyaRajan, Aswathi, Sreekala, Rida, Swathi, Janet, Jily, Revathi, Jobson, Manimaran*** *who have been always participating with my problems and disappointments and rebuilt my confidence at appropriate stages. Also I take this opportunity to thank my batch mates and juniors for their help and support.*

I also express thanks to ***Vanathi, Vijay, Anandhi*** *and* ***Tamilarasan****, Lab technicians for their valuable support and timely help during the course of the entire work.*

With immense pleasure I express my deep gratitude to computer lab technicians, library staff and other lab technicians of KMCH College of Pharmacy, and Store in charge ***Mr.Viji*** *and all those who helped directly and indirectly in every aspect of constructing this work.*

Register No : **261425809**

ABBREVIATIONS

%	Percentage
µg/mg	Microgram per milligram
µg/ml	Microgram per millilitre
µl	Micro litre
µM	Micro molar
AAE	Ascorbic acid equivalent
ABTS	2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
AESP	Aqueous extract of *Sphenodesme paniculata*
CMC	Carboxy methyl cellulose
cm	Centimeter
CNS	Central nervous system
COX	Cyclooxygenase
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EASP	Ethyl acetate extract of *Sphenodesme paniculata*
g	Gram
GAE	Gallic acid equivalent
HPTLC	High performance thin layer chromatography
IC_{50}	Inhibitory concentration 50%
MESP	Methanolic extract of *Sphenodesme paniculata*
M	Molar
mg/kg	Milligram per kilogram
mM	Millimolar
mmol/L	Millimolar per litre
Mo	Molybdenum
MTT	(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide
ng/g	Nanogram per gram
QE	Quercetin equivalent
TAC	Total antioxidant capacity
TPC	Total phenolic content
WHO	World health organisation

CONTENTS

LIST OF TABLE

1. INTRODUCTION

Medicinal plants are the greatest asset to human health and represent a viable treasure for discovering new potential compounds with various therapeutic effects.[1] In addition, factors such as the availability, affordability, and accessibility of medicinal plants have led to their high demand and usage.[2] Secondary metabolites such as alkaloids, glycosides, flavanoids and phenolic compounds generally produced by plants, especially for their defense mechanisms, have been implicated in the therapeutic properties of most medicinal plants. Herbal medicine is a major component in all indigenous system of medicines and our system of medicines like Siddha and naturopathy describes the value of many herbs. Number of drugs commonly used today are of herbal origin. Indeed about 25 percent of prescription drugs dispensed in the united state contain at least one active ingredients derived from plant material .[3]

The world health organization (WHO) estimated that 80 percent of populations of developing countries rely on the traditional medicines, mostly plant drugs for their primary health care needs also modern pharmacopoeias still contain at least 25% drugs derived from plant and many others which are synthetic analogs built on prototype compounds isolated from plants.[4.] Demand of medicinal plants is increasing day by day in both developing and developed countries due to growing recognition of natural products, because no side effects, easily available at affordable prices and sometimes the only source of health care available to the poor. Medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal area of rural and tribal lives of India. Substance derived from the plants remain the basis of a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, treatment of pain asthma and other problems. Example, ephedra is an herb used in Chinese medicine for more than 2000 years to treat asthma and other respiratory problems, similarly many herbs described in ayurveda.

The natural plant products often serve as chemical models or prototypes for the design and total synthesis of new drug entities. The concept of drug design of some of the synthetic molecules has emerged out of their quantitative structural relationship (QSAR) in terms of bio dynamic constituent. For example, the belladonna alkaloids (atropine), quinine, cocaine. Opioids (morphine and codeine) and salicylic acid has been serve as model for design and synthesis of anti cholinergic, anti malarial, benzocain, procain and other local anesthetics and aspirin respectively. Virtually more than13000 plants have been studied during the last five years period.

About 9.5% of new structures obtained from the higher plants were tested for their pharmacological effects. Efforts have been made in last three decades for the development of extraction, isolation, characterization and standardization of phytochemicals on commercial scale pharmaceutical, chemical industries and research institutions. As a result of modern pharmaceutical techniques and pharmacological testing procedures, new plant drugs usually find their values in the medicinal and purified substances.[5]

Natural products have played an important role as new chemical entities (NCEs). Approximately 28% of NCEs between 1981 and 2012were natural products or natural product derived. Another 20% of NCEs during the period were consider natural products mimics, meaning that synthetic compound was derived from study of natural products.[6] Combining these categories research on natural products accounts for approximately 48 % of NCEs reported from 1981-2002.Natural product provide a starting point of new synthetic compound, with diverse structures and often with multiple stereo centers that can be challenging synthetically.[7] When the whole plant is used rather than the extracted constituents, the different parts interact, producing grater therapeutic effect that equivalent dosage of active isolated constituents. In some cases the medicinal value of herb may be due entirely to the combination of the substances and cannot be reproduce by one or two active constituents alone.[8]

1.1 Indian Herbal Market

India herbs play a major in the global market for the herbal products based medicines. Export of herbal materials and medicines can jump from RS 456crore now to RS 10000crore by 2008.[9] Herbal medicines also find market as a neutraceuticals [health food], whose current market is estimated at about $80-250 billion in USA and also in Europe.[10]The factors limiting the rational use of herbal medicine is variation in quality of the product, uncertainty of safety and absence of ambiguous proof of efficacy. Since many herbal medicines have been used successfully over many centuries by indigenous people, the safety is frequently not a big concerned. This faith of the population on naturopathy is an asset due to the fact that many herbal medicines are known to have acceptable side effect and truth that the unearthing of new synthetic drug is a time consuming and expensive matter also. Based on the strong traditional knowledge on the use of plants and therapeutic agents, a rational approach is being developed to use the medicinal plant as a lead for discovery of active molecules with one of the largest reservoir of bio resources.

The criteria for the selection of the plants for herbal drug research for various human ailments are as follows,

- Actual use of medicinal plants in the countries of the region
- Scientific literatures indicating therapeutic efficacy of the plants in certain diseases
- Mention of the plants in early texts as having therapeutic effects
- Use of medicinal plants for therapeutic purpose in countries outside the region.

To evaluate the plant with possible therapeutic effect, the first world congress of clinical pharmacology and therapeutics was held in London 1980.The traditional approach on herbal drug research consist of the following steps,

- Identification of the plant reportedly in use.
- Collection of plant.
- Transport of the plant to the research laboratory
- Storage of plants
- Preparation of the extracts.
- Toxicity studies of the plants in animal models.
- Identification of the extract which having more activities.
- Further fractionation of the active molecules.
- Synthesis of bio active molecules.

Medicinal herbs are significant source of semisynthetic and herbal formulations as in commercial market for various illness. Isolated active constituents are used for applied research. The plant *Sphenodesme paniculata* is locally known as Arambodal / Njarambodal. Ethno botanically, the plants are used by the tribals of pathanamthitta district, kerala, for the treatment of body pain. This plant also used as anti-inflammatory and wound healing agent in part of traditional medicinal formulations that alleviate body pains.[11]The plant *Sphenodesme paniculata*is used in the present study to confirm the biological properties by *invivo* screening method.

1.2 Inflammations

Inflammation is normal protective response to tissue injury caused by physical trauma; noxious chemicals, microbial agents. Inflammation is body effort to destroy or inactivate invading organism remove irritant and set stage of tissue repair, when healing is complete tissue repair is subside .It is a local response of living mammalian tissue to injury due to any agent.The Greek term for inflammation is phlegmone “the firty thing”(pholex =flame).[12] This process

involves changes in blood flow,increased vascular permeability,destruction of tissue by activation and migration of leucocytes by synthesis of reactive oxygen derivatives and loco inflammatory mediators such prostaglandins, leukotrienes and platelet factor induced by phospolipase A_2 and cycloxygenase and lipooxygenase.[13]

1.2.1. Agent causing inflammation

- Infectious agent : Bacteria, viruses and their toxins, fungi.
- Immunological agent : Cell mediated and antigen anti body reaction
- Chemical agents : Organic and inorganic poisons
- Inert materials : Foreign bodies

1.2.2 Signs of inflammation

The inflammation was first described by Celsus who identified cardinal signs of inflammation as:

- Rubor (Redness)
- Tumor(Swelling)
- Calor (Heat)
- Dolor (Pain)
- Function Laesa (Loss Of Function)

Redness of inflammation is due to dilation of vascular bloods in injured area and heat is due to increased blood flow. Swelling occurs due to edema formation caused by fluid accumulation and plasma protein in the extra vascular spaces. Pain inflammation due to increased pressure in the tissue which leads to increased firing of pain afferents in affected area.[14,15]

1.2.3. Mediators of inflammation

- Histamine
- Prostaglandins
- Leukotrienes
- Serotonin
- Lysosome
- Platelet activation factors
- Nitric oxide
- Cytokines
- Bradykinins

1.2.4. Phases of inflammation

- Vasodilatation: Vasodilatation is the first phase of inflammation, caused by increase in vascular permeability result in exudation of fluid from blood into interstitial space
- Exudation :Exudation is the second phase of inflammation; it involves the filtration of leukocytes from blood into tissue
- Emigration of cells : It is the third phase of inflammation; it involves granuloma migration and tissue repair.[16]

1.2.5. Types of inflammation

Inflammation is classified based on the defense capacity of host and duration of response

- Acute inflammation
- Chronic inflammation

a) Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes from the blood into the injured tissues. A cascade of biochemical events propagate and mature the inflammatory response, involving the local vascular system, the immune system and various cells within the injured tissue. It is a short term process which is characterized by the classic signs of inflammation- swelling, redness, pain, heat and loss of function- due to the infiltration of the tissues by plasma and leukocytes.[17]

b) Chronic inflammation is a prolonged inflammationleads to a progressive shift in the type of cells which are present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.[15]

1.2.6 Manifestation of acute inflammation compare to normal

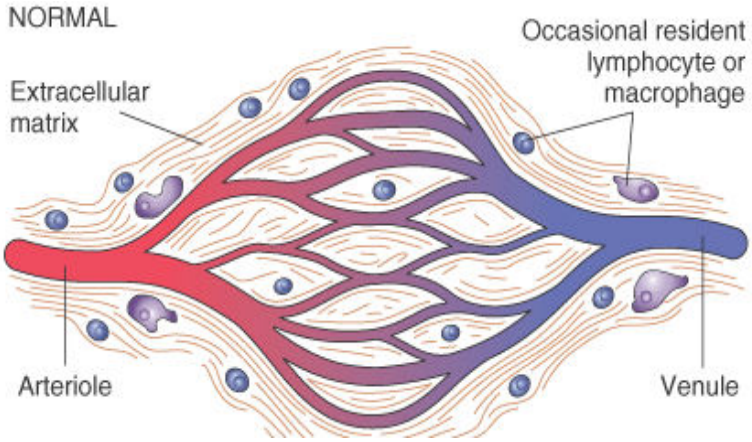


Fig no:1.Normal tissue

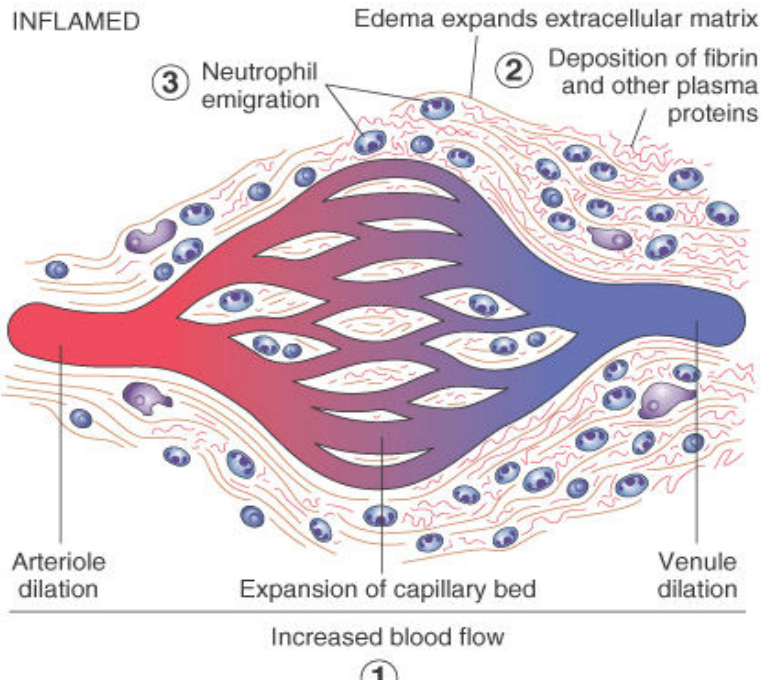


Fig no:2. Inflamedcondition of tissue

1.2.7. Comparison between acute and chronic inflammation

Table no:1

Characteristic	Acute	Chronic
Causative agent	Pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, persistent foreign bodies or auto immune reactions
Major cells involved	Neutrophils, eisonphils, Basophils and mononuclear cells	Mono nuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, eicosanoids	Interferon gamma and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed
Duration	Few days	Up to many months or years
Outcomes	Healing, abscess formation, chronic inflammation	Tissue destruction, fibrosis

1.3. PAIN

Pain is an unpleasant experience which results from both physical and physiological responses to the injury. [18] Pain motivates the individual to withdraw from damaging situations, to protect a damaged body part while it heals and to avoid similar experiences in the future. Most pain resolves promptly once the painful stimulus is removed and the body has healed, but sometimes pain persists despite removal of the stimulus and apparent healing of the body and sometimes pain arises in the absence of any detectable stimulus, damage or disease. Pain is the most common reason for physician consultation in India. It is a major symptom in many medical conditions, and can significantly interfere with a person's quality of life and general

functioning. Psychological factors such as social support, hypnotic suggestion, excitement, or distraction can significantly modulate pain's intensity or unpleasantness.[19]

Pain is mainly a protective mechanism for the body, occurs whenever any tissues are being damaged, and it causes the individual to react to remove the pain stimulus. Typically, it is a direct response to an untoward event associated with tissue damage such as injury, inflammation or cancer, but severe pain can arise independently of any obvious predisposing cause (e.g. trigeminal neuralgia), or persistent long after the precipitating injury has healed (e.g. phantom limb pain). It can also occur as a consequence of brain or nerve injury (e.g. following a stroke or herpes infection).With many pathological conditions tissue injury is the immediate cause of the pain and this result in the local release of a variety of chemical agents which are assumed to act on the nerve terminals either activating them directly or enhancing their sensitivity to other forms of stimulation.[20]

1.3.1. Mediators of pain

Many types of dental pain arise as a result of infection or damage to tissue. Both events initiate an inflammatory response that is intimately linked with pain. The passage of nociceptive impulses generated in the peripheral nerve fibers depends on the release of various neurotransmitters. These neurotransmitters act either peripherally or centrally.

1.3.2.Types of pain

The recent evidence suggests that the pain may be postulated to exist in three different groups of processes, each predominating in different painful disorders. These involve,

1. Nociceptive pain

Nociceptive pain may occur as a secondary phenomenon caused by a non-neurologic source of continuing noxious stimulation in the periphery and may be classified according to the mode of noxious stimulation, the most common categories being "thermal" (heat or cold), "mechanical" (crushing, tearing, etc.) and "chemical" (iodine in a cut, chili powder in the eyes)

2. Neuropathic pain

Neuropathic pain may be generated directly by the disordered or damaged nervous system. Peripheral neuropathic pain is often described as burning, tingling, electrical, stabbing,or pins and needles. Bumping the "funny bone" elicits acute peripheral neuropathic pain

3. Psychogenic pain

In case of psychogenic pain, the psychological factors play an important role in generating or magnifying the pain even when other identifiable neurologic or peripheral causes exist [21]

1.4. Neural pain pathway

- Nociception is the encoding and processing of noxious stimuli in the nervous system that can be measured with electrophysiological techniques. Neurons involved in nociception form the nociceptive system. Noxious stimuli activate primary nociceptive neurons with "free nerve endings" (Aδ and C fibres, nociceptors) in the peripheral nerve. Most of the nociceptors respond to noxious mechanical (e.g. squeezing the tissue), thermal (heat or cold), and chemical stimuli and are thus polymodal Nociceptors can also exert efferent functions in the tissue by releasing neuropeptides substance P (SP), calcitonin gene-related peptide (CGRP)] from their sensory endings. Thereby they induce vasodilatation, plasma extravasation, attraction of macrophages or degranulation of mast cells, etc. This inflammation is called neurogenic inflammation.[22]
- **Nociceptors** are the physiological receptors, activation of this receptor initiate the propagation of pain. Simply sensory receptors which respond to pain and pain full stimuli. When tissue has been damaged, messages are sent along the nerves to spinal cord. widely found in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons, and arterial vessels.[22]

a) Transduction

Transduction is the process by which noxious stimuli are converted to electrical signals in the nociceptors. Nociceptors readily respond to different noxious modalities such as thermal, mechanical or chemical stimuli, but nociceptors do not respondto non-noxious stimuli. Also in contrast to other types of sensory receptors, nociceptors do not adapt–that is, continued stimulation results in continuous or repetitive firing of the nociceptor and, in some cases, continued stimulation actually results in a decrease in the threshold at which the nociceptors respond (ie, sensitization of nociceptors. Neurotransmitters that are produced within the cell body–ie, in the dorsal root ganglia (DRG) are the same at both the central and peripheral ends of

the nerve fiber and are released at both ends, participating in producing the pain signal centrally, as well as in promoting events that lead to additional pain Peripherally.The release of neurotransmitters from the peripheral terminals of the afferent fibers is actually an "efferent" function of these afferent neurons. Peripheral release of neurotransmitter substances lead to the classic "axon reflex", a reflex that does not require the spinal cord–this reflex leads to peripheral changes that are well recognized to contribute to pain.

b) Transmission

Transmission is the second stage of processing of noxious signals, in which information from the periphery is relayed to the thalamus and then to the cortex. Noxious information is relayed mainly via 2 different types of primary afferent nociceptive neurons, which conduct at different velocities.

A-delta fibers are thinly myelinated fibers which conduct in the range of 2 m/s to 20 m/s. All fibers respond to high intensity mechanical stimulation and are therefore termed high threshold mechanoreceptors. Some, but not all fibers also respond to thermal stimuli–the latter are termed mechano–thermal receptors.

C-fibers are non-myelinated fibers that conduct in the range of 0.5 m/s to 2 m/s and transmit noxious information from a variety of modalities including mechanical, thermal, and chemical stimuli–for this reason, they are termed as C-polymodalnociceptors.[23]

1.5. Pain pathways

a) Pain receptors and primary afferents

Nociceptors are receptors in tissues which are activated specifically by painful stimuli. This 'noxious' information is transduced by the receptors into an electrical signal and transmitted from the periphery to the central nervous system along axons. There are two types of nociceptors:_

- High-threshold mechanoreceptors (HTM), which respond to mechanical deformation
- Polymodalnociceptors (PMN), which respond to a variety of tissue-damaging inputs:

These inflammatory mediators bathe the nociceptors, activating and sensitizing them. Prostaglandins and bradykinin sensitize nociceptors to activation by low-intensity stimuli. Histamine and 5-HT cause pain when directly applied to nerve endings. Hydrogen ions and 5-HT act directly on ion channels on the cell membrane, but most of the others bind to membrane

receptors and activate second-messenger systems via G proteins. Nociceptors are therefore the free nerve endings of nerve fibres. There are two main fibre types: Ad and C fibres. These primary afferent nerve fibres have cell bodies in either the dorsal root ganglia or trigeminal ganglion and terminate in the dorsal horn of the spinal cord. Although all pain fibres terminate in the dorsal horn, their route to this end-point varies. Most enter the dorsal horn in the ventro-lateral bundle of the dorsal root. They travel just lateral to the larger diameter myelinated Ab fibres, which respond to non-painful stimuli such as vibration and light touch. However, 30% of the C fibres enter the spinal cord via the ventral root. Once they have entered the spinal cord the nerve roots may bifurcate into ascending and descending branches, which can enter the dorsal horn one or two segments higher or lower than the segment of origin.[24]

b) Ascending tracts

Second-order neurons ascend to higher centres via the contralateral spinothalamic and spinoreticular tracts, which are located in the anterolateral white matter of the spinal cord.

- **The brain**

The brain is the main structure involved in the way we try to make sense of pain the brain help us to judge where is the pain is coming from ,which reaction to take towards the brain and how we experience pain the brain is also the structure where thoughts, anxieties and emotions about pain may start.

The thalamus is the key area for processing somatosensory information. Axons travelling in the lateral and medial spinothalamic tracts terminate in their respective medial and lateral nuclei and from here neurons project to the primary and secondary somatosensory cortices, the insula, the anterior cingulate cortex and the prefrontal cortex. These areas play various roles in the perception of pain and also interact with other areas of the brain, for example the cerebellum and basal ganglia (which are areas more traditionally known to be associated with motor function rather than pain).[25]

c) Descending tracts

These pathways (see Figure 1) have a role in the modulation of pain. Noradrenaline and 5-HT are the key neurotransmitters involved in descending inhibition. Two important areas of the Brain stem are involved in reducing pain; the periaquaductal grey (PAG) and the nucleus raphe magnus (NRM).[22]

C.1. PAG

This region surrounds the cerebral aqueduct in the midbrain and is important in the control of pain. Electrical stimulation of the PAG produces profound analgesia and injection of morphine

here has a far greater analgesic effect than injections anywhere else in the central nervous system (CNS). The PAG receives inputs from the thalamus, hypothalamus and cortex and also

collaterals from the spinothalamic tract. PAG (anti-nociceptor) neurons excite cells in the NRM that in turn project down to the spinal cord to block pain transmission by dorsal horn cells.

C.2. NRM

A second descending system of serotonin-containing neurons exists. The cell bodies of these neurons are located in the raphe nuclei of the medulla and, like the noradrenalin-containing neurons, the axons synapse on cells in lamina II. They also synapse on cells in lamina III. Stimulation of the raphe nuclei produces a powerful analgesia and it is thought that the serotonin released by this stimulation activates inhibitory interneurons even more powerfully than noradrenaline and thus blocks pain transmission.[24,25]

Brainstem neurons may control nociceptive transmission by:

- direct action on dorsal horn cells
- inhibition of excitatory dorsal horn neurons
- Excitation of inhibitory neurons.

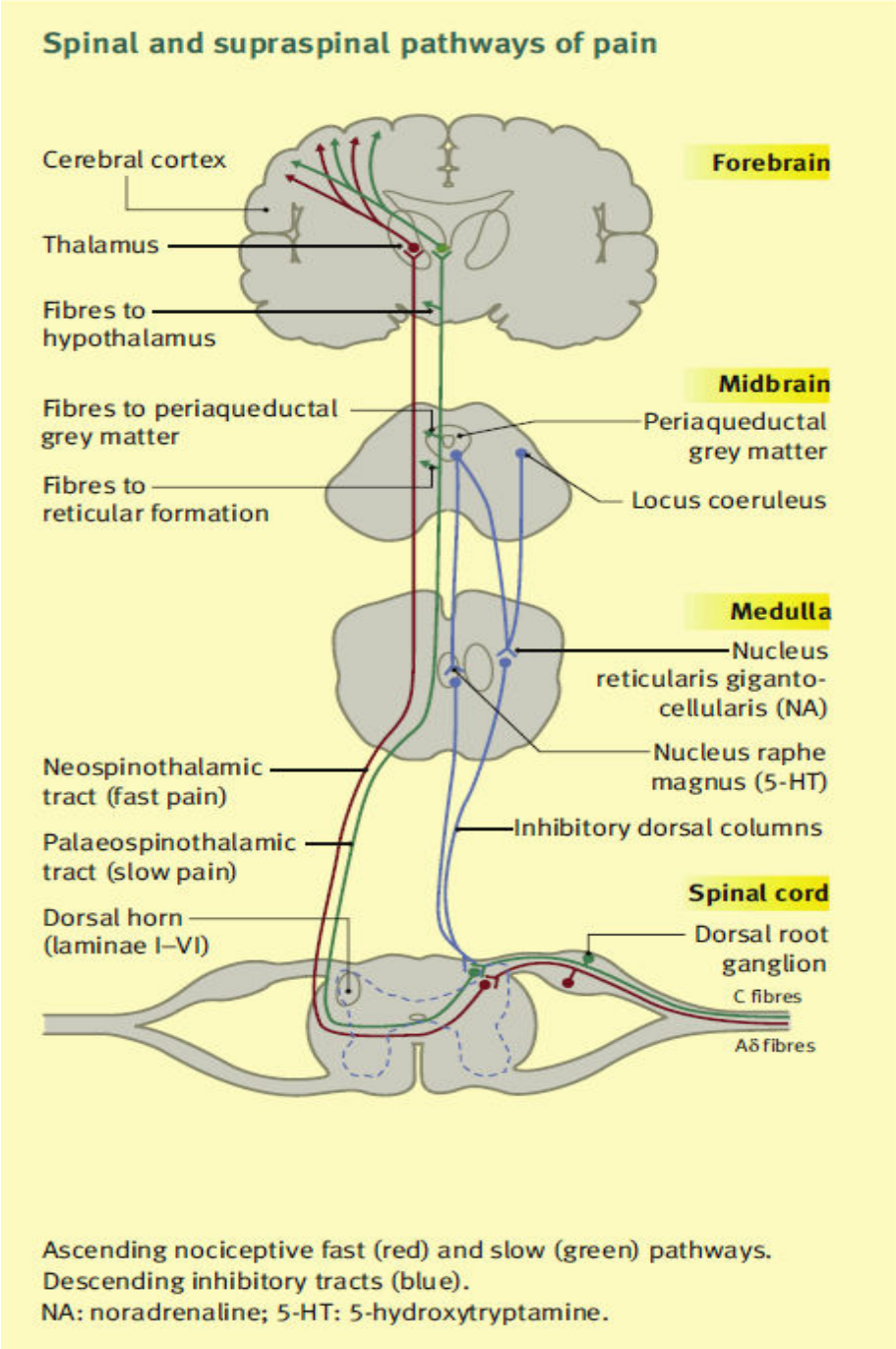

Spinal and supraspinal pathways of pain
Cerebral cortex
Thalamus
Fibres to hypothalamus
Forebrain
Fibres to periaqueductal grey matter
Fibres to reticular formation
Midbrain
Periaqueductal grey matter
Locus coeruleus
Medulla
Nucleus reticularis giganto-cellularis (NA)
Nucleus raphe magnus (5-HT)
Neospinothalamic tract (fast pain)
Palaeospinothalamic tract (slow pain)
Inhibitory dorsal columns
Spinal cord
Dorsal horn (laminae I–VI)
Dorsal root ganglion
C fibres
Aδ fibres
Ascending nociceptive fast (red) and slow (green) pathways.
Descending inhibitory tracts (blue).
NA: noradrenaline; 5-HT: 5-hydroxytryptamine.

Fig no:3

2. REVIEW OF LITERATURE

The literature revealed the following activities have been reported for the plant ***sphenodesme paniculata***

1. AsutoshGhosh has listed *Sphenodesmepaniculata* among other plants in the census of the climbers of North Andaman and Nicobar Islands.
2. Binu S, in his study "Medicinal plants used for treating body pain by the tribals in Pathanamthitta district, Kerala, India" has reported the use of the plant in the treatment of pains.[27]
3. Details of toxic effects are not reported other than the lethal dose values as given in the Indian Journal of Experimental Biology.

2.1.Plant Profile

***Sphenodesme paniculata* (Clarke)**

Synonym **:***Sphenodesme paniculata*

Common name **:**Njarambodal /Arambodal
Family **:**symphorematacеae (based on APG:Lamiaceae)

Order **:**Lamiales

Genus **:**sphenodesme

Species **:***paniculata*

Vernacular names

Malyalam :Njarambodal or Aramboda

Habitat

Sphenodesme paniculata (Clarke) is a climber found in the Semi-evergreen and evergreen forests, also in sacred groves of Western and Eastern ghats and is endemic to South India. Plant belongs to Verbenaceae family.

Fig no:4.*Sphenodesme paniculata*

Description: *Sphenodesme paniculata is a* climbing or scandent shrubs, branchlets sub-tetragonous, canescent.

Leaves

leavesto 14 x 8 cm, broadly elliptic, acute, base obtuse or acute, entire, glabrous above and tomentose below, nerves 6-8 pairs, prominent, nervules reticulate; petiole 1.5 cm long. Panicles terminal, golden-brown tomentose, 20-30 cm long; involucral bracts 6, obovate, obtuse, subequal, to 2.5 x 1 cm.

Flowers

5-15 together; calyx densely tomentose, 6 mm long, 3-6-lobed, lobes ovate, acute; corolla white, 1 cm long, funnel-shaped, 5 or 6-lobed, lobes oblong, tomentose; stamens 5 or 6, included; ovary 2-celled, ovule 2 in each cell, style short, stigma 2-lobed. Drupe globose, enclosed by the calyx, 12-seeded

Flowering and fruiting

December-April

2.1.Traditional medicinal uses

- It has been used in the traditional medicine as a galactogogue and analgesic. The plant is also a part of medicinal formulations that alleviate body pains**.**
- In another study it has been reported that the plant is used in the treatment of pains and wound healing by the tribals of some parts of Kerala. [27]

2.3. Some of the plants reported analgesic and anti-Inflammatory activity

Table no:2

Si no	**Botanical Name (Common Name)**	**Family**	**Part used**	**Chemical constituent**	**Activity**	Ref
1	*Mitragynaparvifolia (kadam)*	Rubiaceae	Fruits	pyroligneous acid, methyl acetate, ketones and aldehydes	Anti inflammatory, Analgesics	28
2	*Nyctanthes arbor - tristis (Shefali)*	Oleaceae	Bark	flavonol glycosides, β - sitosterol, nyctanthic acid.	Anti inflammatory, Analgesics	29
3	*Phyllanthusniruri (Gulf leaf)*	Phyllanthaceae	Whole plant	Flavonoids, sterols, alkaloids, phyllanthin, hypophyllanthin	Anti inflammatory Analgesics	30
4	*Sterculiafoetida (Janglibadam)*	Sterculiaceae	Seed	Fat, cycloprenoid fatty acids.	Anti inflammatory Analgesics	31
5	*Holarrhenaantidysenterica (Indrajao)*	Apoynaceae	Bark	Alkaloid, Tannins &Flavanoids	Anti inflammatory Analgesics	32
6	*Tridexprocumbens (Ghamra)*	Asteraceae	leaves	flavonoids, procumbentin and quercetin,β- sitostero	Anti inflammatory Analgesics	33
7	*Cissusrependa (Panibel)*	Vitaceae	Root and stem	Alkaloids, glycosides, saponins, tannins.	Anti inflammatory Analgesics	34
8	*Kaempferia galangal (Aromaticginger)*	(Zingiberaceae)	fresh rhizome	ethyl-p-methoxycinnamate, methylcinnamate,Carvone etc	Antiinflammatory, Analgesics	35
9	*Tanacetumartemisioides (Paloyo Zoon)*	Asteraceae	whole plant	Flavonoids	Antiinflammatory, Analgesics	36
10	*Hedyotispuberula*	Rubiaceae	whole	Iridoid glycosides	Antiinflammatory,	37

	(Surbuli)		plant		Analgesics	
11	*Eucalyptus citriodora (lemon eucalyptus)*	Myrtaceae	essestial oil	Terpenes, alkaloids, flavonoids, tannins, eucalyptol.	Antiinflammatory, Analgesics	38
12	*Chococcabrachiata*	Rubiaceae	Root	Steroids, phenolic compounds, ligans	Antiinflammatory, Analgesics	39
13	*Cynarascolymus (Globe artichoke)*	Asteraceae	Leaves	Sesquiterpenes, flavone glycosides, volatile oil.	Antiinflammatory, Analgesics 33	40
14	*Elephantopusscaber (Elephant foot)*	Asteraceae	Leaves	Glycosides, stigmasterol, deoxyelephantopin	Antiinflammatory, Analgesics	41
15	*Cissusquadrangularis (Hadjod)*	Vitaceae	whole plant	flavonoids, coumarins, steroids	Antiinflammatory, Analgesics	42
16	*Cissampelospareira (Akanadi).*	Menispermacea e	Aerial parts	Alkaloids, flavoncurine, volatile oil, quercitol	Antiinflammatory, Analgesics	43
17	*Thesiumchinense (bairuicao)*	Santalaceae	Leaves	Flavanoids, glycosides, essential oils, Alkaloids, Steroids	Antiinflammatory, Analgesics	44
18	*Rubiacordifolia (Indian Madder)*	Rubiaceae	root	Purpurin, xanthin, glycosides, manjisthin, resins	Antiinflammatory, Analgesics	45
19	*Solanumtrilobatum (Alarka)*	Solanaceae	root	Tannins, saponins, flavonoids, cardiac glycosides.	Antiinflammatory, Analgesics	46
20	*Mangiferaindica (Am)*	Anarcardiaceae	Leaves	Flavonoids, polyphenolics, triterpenes, tannins	Antiinflammatory, Analgesics	47
21	*Asystasiadalzelliana (Lavana-valli)*	Acanthaceae	Whole plant	Alkaloids, saponins, cardiac glycosides, flavanoids, anthraqui	Antiinflammatory, Analgesics	48
22	*NothospondiasStudtii*	Simaroubaceae	leaves	Alkaloids	Antiinflammatory, Analgesics	49

3. AIM AND OBJECTIVES

3.1 AIM

Sphenodesm epaniculata (Clarke) isa Climbing or scandent shrubs, with traditionally medicinal plants used for treating wound and body pain by the tribals in Pathanamthitta district, Kerala, India. Current literature study revealed that the anti-inflammatory and analgesics activity of Sphenodesmepaniculata (Clarke) have not being studied. Hence the aim of the present study is to evaluate analgesics, anti-inflammatory and cytotoxicity studies of methanolic stem extract of *Sphenodesme paniculata* (Clarke)

3.2 OBJECTIVES

- **Collection of the plant**
- **Authentification**
- **Extraction of stem**
- **Phytochemical analysis**
  - Qualitative analysis
  - Quantitative analysis
- ***In-vitro* antioxidant studies**
  - DPPH
  - ABTS
- **Cytotoxicity activity**
  - HeLa cell line
- **Anti microbial activity**
  - Disc diffusion method
- ***Invivo* studies**
  - Acute toxicity study
  - Evaluation of the analgesic, anti inflammatory activities of the methanolic stem extract of *Sphenodesme paniculata*

3.2. PLAN OF WORK

1. Literature review

2. Selection, collection and authentication of plant material

3. Extraction of plant

4. Preliminary phytochemical analysis.

- Qualitative chemical test
- Estimation of total phenol
- Estimation of total flavanoids
- Estimation of flavanoida by HPTLC

5. ***In-vitro*** **antioxidant activity**

- DPPH
- ABTS
- Phosphomolybdenum assay

6. *In-vitro* anti-microbial activity

- Disc diffusion method

7. Acute toxicity study

8. Pharmacological study

- Screening of anti-inflamatory activity
  - Carrageenan-induced paw oedema model
  - Histamine induced paw edema
- Screening of analgesics activity
  - Hot plat method
  - Writhing test

9. Statistical Analysis.

4. EXPERIMENTAL WORK

4. 1. Materials and Methods

4.1. 1.Chemicals and reagents

Standard rutin, gallic acid, quercetin were purchased from Natural Remedies Pvt. Ltd, Bangalore, Methanol HPLC grade (SD Fine Chemicals, Mumbai, India) was used as a solvent for the preparation of standards and samples. Toluene, ethyl acetate, formic acid and methanol (CDH Labs, Mumbai, India) were used as mobile phase for HPTLC analysis. 2- amino ethyl diphenyl borinate was purchased from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. All solutions used for the analysis were filtered through 0.22 μm syringe- driven filter (HIMEDIA, Mumbai, India). Sulphuric acid, methanol, petroleum ether, 2.2'- azinobis 3- ethyl benzothiazoline 6-sulfonate and Folin- Ciocalteu's reagent was acquired from HiMedia Laboratory Pvt. Ltd, Mumbai, India.. Potassium ferricyanide, trichloroacetic acid, ferric chloride, sulphuric acid, sodium phoshphate, ammonium molybdate, sulphuric acid, sodium niroprusside, phosphoric acid, sulphanilamide, phosphoric acid, ethylene diamine hydrochloride, potassium persulfate, sodium carbonate was acquired from SRL Pvt. Ltd, Mumbai, India.

4.1.2. Instruments

Plant extracts were made by hot percolation using soxhlet extractor (Omega, Mumbai, India). Extracts were dried under vacuum by using rotary evaporator (Buchi R-114, Switzerland). For HPTLC analysis of flavanoids, a CAMAG HPTLC system (Muttenz, Switzerland) equipped with a Linomat IV sample applicator was used. Extracts were applied on aluminum backed TLC plates (20×10cm) pre-coated with silica gel $60F_{254}$ (Merck, Darmstadt, Germany). GC-MS (Agilent 19091S-433: 1548.52849), CAMAG twin trough chamber (vertical development) was used for developing the TLC plates.

4.1.3. Collection of plant material

The plant, *Sphenodesme paniculata* (Clarke) was collected from the botanical garden of Arya Vaidya Sala, Kottakkal and was authenticated by a scientist at the Centre for Medicinal Plant Research under the Kottakkal Arya Vaidya Sala on 31 December, 2014.

4.2. Preparation of different extracts

The stem of *Sphenodesme paniculata* (Clarke) was selected and extractions were carried out with ethyl acetate, methanol and distilled water.

4.2.1. Preparation of ethyl acetate and methanolic extract

The coarse dried stem powdered was initially defated with petroleum ether(60-80)followed by same powder extracted with ethyl acetate and methanol respectively by successive fraction method.Each extraction was carried out in soxhlet apparatus for about 48 hr. Ethyl actate and methanolic extracts were pooled and evaporated to dryness under reduced pressure at 40°C in a rotary evaporator. The ethyl acetate and methanolic extracts were named as EESP and MESP (Ethanolic and Methanolic extract of *Sphenodesme paniculata*).

4.2.3. Preparation of aqueous extract

The aqueous extract was prepaid by hot extraction process, dried plant material was boiled with distilled water ten times with specific time intervals for 2 days .The decoction was filtered and evaporated on water bath at 50-60°C. The resultant aqueous extracts were named as AESP (aqueous extracts of *Sphenodesme paniculata*).

4.3. Phytochemical screening of extracts [48]

4.3.1. Preparation of test sample

About 1g of each extracts were dissolved in 10 ml of distilled water to produce a concentration of 100 mg/ml and the presence of various phytochemicals were analysed

1. Test for alkaloids

a) Mayer's test

Small quantities of the each extract was separately treated with few drops Mayer's reagent (mercuric chloride and potassium iodide). Formation of yellowish buff colour precipitate indicates the presence of alkaloids.

b) Dragendroff's test

To 2-3 ml of each extracts, few drops of the Dragendorff's reagent (Sodium iodide, basic bismuth carbonate, glacial acetic acid and ethyl acetate) were added. Development of orange brown precipitate indicates the presence of alkaloids.

c) Wagner's test

Small quantities of the each extracts were treated with Wagner's reagent (solution of iodine in potassium iodide) and reddish brown precipitates indicate the presence of alkaloids.

d) Hager's test

Small quantities of each extracts were treated with Hager's reagent and development of reddish brown precipitate indicates the presence of alkaloids.

2.Test for flavonoids

a) Ferric chloride test

To a small quantity of each extracts few drops of neutral ferric chloride solution were added. Formation of blackish red colour indicates presence of flavonoids.

b) Alkaline reagent test

To the each extracts, few drops of sodium hydroxide solution were added. Formation of an intense yellow color, which turns to colorless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavanoids.

3. Test for phytosterols and triterpenoid

a) Libermann – Burchard test

Small quantities of each extracts were treated with few drops of acetic anhydride, followed by a few drops of concentrated sulphuric acid. A brown ring was formed at the junction of two layers and the upper layer turns green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

b) Salkowski test

A small quantity of the each extracts were treated with chloroform and few drops of concentrated sulphuric acid and allowed to stand for few minutes. Yellow colour at the lower layer indicates the presence of triterpenoids.

4. Test for reducing sugars

a) Benedict's test

To the each test samples, equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) were mixed in a test tube and heated for few minutes. Formation of Brick red Precipitate confirmed the presence of sugars.

b) Fehling's test

Equal volume of Fehling's- A [copper sulphate in distilled water] and Fehling's- B [potassium tartarate and sodium hydroxide in distilled water] reagents were mixed in a test tube and boiled for one minutes. To this 1 ml of sample was added and heated for few minutes. Formation of brick red precipitate confirmed the presence of sugars.

c) Barfoed's test

To a few ml of the each test samples, 5 ml of Barfoed's reagent was added and boiled. Formation of red precipitate of copper oxide indicates the presence of monosaccharide.

5. Test for tannins

a) Lead acetate test

Mixed 1ml of test samples with 10% aqueous lead acetate solution. Development of yellow colour precipitate indicates the presence of tannins**.**

b) Treated 1 ml of each test sample with 1 mL of ferric chloride solution. Dark blue or greenish black colour was produced, which indicated the presence of tannins.

6. Test for glycosides

a) Killer killiani test

Extracts (2ml) were dissolved in acetic acid containing trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of two liquids, a reddish brown color formed, which gradually became blue colour due to the presence of glycosides.

b) Legal's test

Dissolved 2ml of each extracts in pyridine; sodium nitroprusside solution was added to it and made alkaline. Pink red colour was formed which indicates the presence of glycosides.

c) Baljet test

Extracts (2ml) were added with sodium picrate solution. Formation of yellow to orange colour indicating the presence of glycosides.

d) Borntrager's test

Added 1 ml of diluted sulphuric acid was added to 2 ml of extracts. The mixture was boiled, filtered and the filtrate was extracted with ether or chloroform. Then organic layer was separated to which ammonia was added, pink, red or violet colour was produced in organic layer, which indicates the presence of glycosides.

7. Test for protein and amino acid

a) Biuret test

To 1 ml of each test sample, 1 ml of 40 % sodium hydroxide and 2 drops of 1% copper sulphate was added. Formation of violet colour indicates the presence of proteins.

b) Ninhydrin test

To 1 ml of each test sample, 2 drops of freshly prepared 0.2% ninhydrin reagent was added and heated. Development of blue colour indicates the presence of proteins, peptides or amino acids.

8.Test for saponins

a) Foam test

About 1 ml of each test sample is diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for 3 minutes. Foam of 1 cm after 10 minutes indicates the presence of saponins.

b) Froth test

To 5 ml of the each test sample, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. Froth was formed which shows the presence of saponins.

9. Tests for Phenols

a) Ferric chloride test

To 1 ml of the each extracts, 2 ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green colour indicates the presence of phenols.

b) Lead acetate test

The extracts were diluted with 5 ml of distilled water and to this few drops of 1% aqueous solution of lead acetate was added. A yellow colour precipitate was formed which indicates the presence of phenols.

4.4.Estimation of total phenolic contents

The TPC of all extracts were determined by Folin-ciocalteau assay with some modifications Briefly, 1 ml of different concentrations of samples was taken in a 2 ml centrifuge tube and added 0. 5ml folins reagent (1:10 diluted with distilled water) and 0.4ml sodium carbonate (1M), mixed and allowed to stand for 15 min at room temperature. Absorbance was measured at 765 nm. The blank was prepared in similar manner without sample/standard. Calibration curve was plotted using gallic acid as standard (10, 20, 30, 40, 50µg/ml). The results were expressed as milligram of gallic acid equivalents (GAE) per gram of extract.[49]

4.5. Estimation of total flavanoid content

Total flavanoids content of the extracts were estimated by aluminium chloride colorimetric assay with some modifications. An aliquot (1ml) of diluted sample or standard solution of quercetin (10, 20, 30, 40 and 50 µg/ml) was mixed with 50 µl of $NaNo_2$ in 2 ml microcentrifuge tube. After 6 min, 50 µl 1M potassium acetate solutions were added to the mixture. Distilled water was added to bring the final volume to 2 ml, and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Then filtered all the solutions using whatmann filter No1 paper. Absorbance of the mixture was determined at 510 nm against prepared blank. Blank was prepared in the above manner omitting sample/standard. All values were expressed as milligrams of quercetin equivalent per 1g of sample.[50]

4.6. Densitometric determination of polyphenols by HPTLC

High performance thin layer chromatography is a suitable quality assessment tool quantification of analyte at nano levels can be estimated. Numerous samples can be run in a single analysis there by it will reduce the analytical time. With HPTLC, the same analysis can be viewed single and different wavelength of light there by providing a more complete profile of the plant then it is typically observed with more specific types of analysis. HPTLC has proved a very useful technique because of its high sample through put, low operating cost and need for minimum sample clean-up.

4.6.1. Preparation of standard solutions

The standard solution of polyphenols was prepared by dissolving accurately weighed 1.0 mg each of gallic acid, rutin and quercetin in 1.0 ml of methanol (HPTLC grade) as stock solution and stored at 4 ˚C. These standards were further diluted as per the requirement to a desired concentration for quantification.

4.6.2. Preparation of sample solutions

Accurately weighed 100 mg of methanolic extract was dissolved in 10ml volumetric flasks with 5ml methanol (HPTLC grade). The solution was sonicated for 10 min and then made up to 10 ml with methanol. The solution was filtered through whatman filter paper before applying to HPTLC plate.

4.6.4. Estimation of different markers

A 10 µL each of sample solution was applied in triplicate on silica gel 60 F_{254} plates with CAMAG Linomat-5 Automatic Sample Spotter. The peak areas and absorption spectra were recorded.

4.6.5. Instrument

CAMAG Linomat 5

4.6.6. Application parameter

Spray gas	:	Inert gas
Sample solvent type	:	Methanol
Dosage speed	:	150 nl/s
Predosage volume	:	0.2 ul
Syringe size	:	500 µl
Number of tracks	:	8
Application position Y	:	10.0 mm
Band length	:	6.0 mm

4.6.7. Development parameters:

Chamber type	:	Twin Trough Chamber 10×10 cm
Mobile phase	:	Toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4)

Solvent front position	:	80.0 mm
Volume	:	10.0 ml
Drying device	:	Oven
Temperature	:	60 °C

Time: 5 Minutes

4.6.8. Detection parameters

Detector	:	CAMAG TLC Scanner
Position of first track	:	10.0 mm
Distance between tracks	:	10.0 mm
Scan start position	:	5.0 mm
Scan end position	:	75.0Scanning speed: 20 mm/s
Data resolution	:	100
Wavelength	:	254
Lamp	:	D2 & W
Measurement Type	:	Remission
Measurement Mode	:	Absorption μm/step mm

4.6.9. Procedure

The samples and standard were spotted in the form of bands with a Camag micro litre syringe on pre-coated silica gel 60F 254 coated aluminum plate (10×10 cm with 0.2 mm thickness) using a camag linomat 5 applicator. The plates were pre-washed with methanol and activated at 60˚C for 5 min prior to chromatography. The sample loaded plate was kept in TLC twin trough developing chamber after chamber saturation with respective mobile phase. The optimized chamber saturation time for mobile phase was 10 min at room temperature. Linear ascending development was carried out and the plate was developed in the respective mobile phase up to 7 cm. The developed plate was dried in oven at 60˚c for 5 min to identify the compact bands. The photo documentation was performed and the plate was scanned at 254 nm using densitometer (camag scanner 3) and operated by win CATS Planar chromatography manager.

4.7. IN VITRO ANTIOXIDANT STUDY

Methanolic exract of *sphenodesme paniculata* was subjected to estimate the free radical scavenging ability by DPPH and ABTS assay method

4.7.1DPPH radical scavenging activity

a) Principle

The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH* with an odd electron gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH*, which is a stable free radical; it becomes paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPHH and as a result, the absorbance is decreased from the DPPH. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured. More the decolorization more is the reducing ability. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, it gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet colour and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability .[51]

b) Procedure

The free radical scavenging activities of the sample was measured in terms of hydrogen donating or radical scavaenging ability using the stable Diphenyl-2-picrylhydrazyl (DPPH) radical (Blois method).0.3mM solution of DPPH solution in methanol was prepared and 1ml of this solution added to 1 ml various concentration of sample and reference compound(10,20,30,40,50 and 60µg/ml)were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm against the blank. Ascorbic acid at different concentrations was considered as standard. Control reaction was carried out without test sample .all the test were performed in triplicate order to get the mean values. DPPH radical scavenging activity % was calculated for the samples and the standard using following formula .[52]

$$\%\text{Scavenging Activity} = \{(Ab_c - Ab_{s)} \div Ab_c\} \times 100$$

Where, Ab_c is absorbance of control and Ab_s is absorbance of sample

4.7.2. ABTS radical scavenging assay

a) Principle

2, 2-azinobis (3-ethyl benzoline-6- sulfonic acid) or ABTS is a chemical reagent used to observe the reaction kinetics of specific enzymes. The formal reduction potential for ABTS is high enough for it to act as an electron donor for the reduction of reactive oxygen species such as molecular oxygen and hydrogen peroxide, particularly at the less extreme pH values encountered in biological catalysis. Under this condition, the sulphonate groups are fully de-protonated and the mediator exists as a "dianion".

$$ABTS + e \longrightarrow ABTS^{-}$$

$$ABTS^{-} + e \longrightarrow ABTS^{2-}$$

b) Procedure

This assay is based on the inhibition of the absorbance of the radical cation 2, 2 azinobis 3-ethylbenzothiazoline 6-sulfonate; ABTS (Katalinic, 2005). ABTS reagent was dissolved in water to a 7mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 24 h before use. The radical was stable in this form for more than 2 days when stored in the dark at room temperature. Initially, ABTS radical ssolution was diluted with phosphate buffer saline (PH 7.4) to an absorbance of 0.70 (± 0.04) at 734 nm and equilibrated at 30˚C.Added 950 µl of diluted ABTS radical solution to 50µl of various concentrations of sample extracts or series concentration of quercetin standard was incubated for 6min in a microcentrifuge tube at 30˚C. The decrease in absorbance at 734 nm was determined exactly at 6 min after initial mixing for all samples. All measurements were performed in triplicate. The percentage inhibition of ABTS by the samples were calculated according to the formula. [53, 54]

$$\% \text{ Inhibition} = \{(A_{c\,(0)} - A_{A\,(t)})/A_{A\,(0)}\} \times 100$$

Where $A_{c\,(0)}$ is the absorbance of control at t=0 min and $A_{A\,(t)}$ is the absorbance of anti oxidant (samples extracts or ascorbic acid standard) at t= 6 min

4 .7.3.Total antioxidant cappacity

a) Principle

This method is based on the reduction of phosphomolybdic acid to phosphomolybdenum blue complex by sodium sulfide. The obtained phosphomolybdenum blue complex is oxidized by the addition of nitrite and this causes a reduction in intensity of the blue color .

b) Procedure

The antioxidant activity of sample was evaluated by the phosphomolybdenum method . Various concentrations of methanolic extract of *spenodesme paniculata* sample (200µl) combined with 2 mL of 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The resulting solution was incubated in water bath at 95°C for 90 min. After incubation, the mixture was cooled to room temperature and the absorbance of the mixture was measured at 695 nm against blank. The tests were performed in triplicates. All the results were reported in mean values expressed as g of ascorbic acid equivalents/100 g extract.[55]

4.8. In *vitro* cytotoxicity studies of crude extracts [56]

4.8. 1. Materials

HeLa cell line (National Centre for Cell Science) (NCCS), Pune, India), Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and MTT assay kit, trypsin EDTA, Penicillin and Streptomycin and DMSO (Sigma-Aldrich Co. LLC, St. Louis, MO, USA), Trypan blue solution and, galactosamine and absolute ethanol (Himedia Lab Pvt. Ltd., Mumbai, India).

4.8.2. Apparatus

Tissue culture flasks, 96 and 24 well micro culture plates, eppendorf tube, inverted microscope, serological pipette, heamocytometer (Himedia Lab Pvt. Ltd., Mumbai, India), laminar flow hoods (Khera instrument, New Delhi, India), CO_2 incubator (NuAire, USA), Water bath, Deepfreezer (-20°C).

4.8. 3. Maintenance of cell lines

HeLa cell line were grown in 25 cm^2 tissue culture flasks containing minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin /streptomycin at 37°C in a CO_2 incubator in an atmosphere of humidified 5% CO_2 and 95% air. The cells were maintained by routine sub culturing in 25 cm^2 tissue culture flasks.

4.8.4. Method for passaging the cells

All the reagents were brought to room temperature before use. Media was removed from the 80-90% confluent flasks by 10 ml serological pipette. Cells in T-75 flask were washed with 10 ml of PBS. Two milliliters of 0.1% trypsin EDTA was added to the flask. The flask was kept at 37°C in the CO_2 incubator for 2-3 min and was observed under microscope for detachment. Six milliliters growth medium was added to the flask for inhibition of trypsin action and re suspended properly by pipetting. The cell suspension was collected in 15 ml falcon tube, and then centrifuged at 1200 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 3 ml of complete medium. Cells were counted, and then 0.2-0.4 million cells were kept in T-25 flask for growing. The flasks were incubated in CO_2 incubator at 37 °C and the cells were periodically monitored for any morphological changes and contamination. After the formation of 80- 90% confluent monolayer, the cells were further utilized.

4.8.5. Principle of MTT Assay

The principle of cytotoxicity estimation is based on ability of the mitochondria of liver cells to metabolize MTT to purple coloured formazan. The outcome of assay depends on both the number of cells present and the mitochondrial activity per cell. Mitochondrial enzyme succinate dehydrogenase cleaves tetrazolium salt 3-(4, 5-dimethyl thiazole-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) into a purple coloured product (formazan). The number of cells is proportional to the extent of formazan production by the cells.

4.8. 6. Preparation of sample solutions

The 10 mg of crude methanolic extract of *spenodesme paniculata* was dissolved in DMSO and the volume was made up to 10ml with DMEM to obtain a stock solution of 1mg/ml concentration and stored at -20°C. Further dilutions were made to obtain different concentrations ranging from 12.5 to 200µg/ml with Dulbecco's modified eagle's medium and used for in-vitro analysis. A residue of serum metabolites was suspended in DMSO and the volume was made up to 1ml with MEM to get a stock solution and kept in freezer at -20°C.

4.8.7. Effect of *Sphenodesme paniculata* extracts on HeLa cell viability

Procedure: Cell viability was determined by a colorimetric MTT assay as described by Sylveste

1. The monolayer cell culture of HeLa cell line was detached by trypsinization.
2. HeLa cells were cultured in 24-well plates at a seeding density of 1×10^5cells/well
3. After 24 h incubation, the cells were treated with ethyl acetate,methanolic, and aqueous extracts (12.5 -200 µg/ml) in separate wells incubated for 48 h in CO_2incubator.
4. After 48 h, the cells were washed and incubated for 1 h with MTT (20 µl of 5mg/ml of MTT in PBS) was added to each well.
5. Formation of formazan crystal was observed under a microscope. In case, crystal formation was not proper, incubation was continued for an hour more.
6. Media was removed and the remaining formazan crystals were dissolved in 100 µl of DMSO in each well. The cell culture plate was kept on a shaker for 15 min. The absorbance was recorded by ELISA reader at 540 nm.

The percentage cytotoxicity was calculated by the following formula:

% cytotoxicity = [(absorbance of control-absorbance of test)/absorbance of control]×100

4.9.ANTI-MICROBIAL ACTIVITY.

4.9.1.Anti –bacterial study[57]

The ethanolic methanoilc and aqous extract of *sphenodesme paniculata* was subjected to screen anti bacterial activity against five gram positive and gram negative bacteria

4.9.1.1. Preparation of inoculums

The inoculums for the experiment were prepared in fresh Nutrient broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards. The turbidity of the culture may be adjusted by the addition of sterile saline or broth (if excessive or by further incubation to get required turbidity).

4.9.1.2. Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or dry heat (only for wooden swabs) by packing the swabs in culture tubes, papers or tins etc.

4.9.1.3. Sterilization of forceps

Sterilize forceps by dipping in alcohol and burning off the alcohol

4.9.1.4. Experiment

The standardized inoculums is inoculated in the plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing by pressing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. Each Petri dish is divided into 4 parts, in each part samples disc such as AQESP, EAESP, and MEESP, (200µg) discs (discs are soaked overnight in sample solution) and Std Ciprofloxacin 10µg, are placed in the 4 th part of the plate with the help of sterile forceps. Then Petri dishes are placed in the refrigerator at 4º C or at room temperature for 1 hour for diffusion. Incubate at 37 º C for 48 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.[58,59]

Table 3: Bacterial strain used for the study with NCIM

Sl no	Organism	Strain	NCIM
1	Gram + ve bacteria	*Staphylococcus lentus*	2169
2		*Staphylococcus albus*	*2178*
3		*Staphylococcus aureus*	*2079*
4		*Bacillus subtilis*	2063
5		*Bacillus lentus*	*2018*
6	Gram –ve bacteria	*Vibrio cholerae*	*1738*
7		*Corynebacterium*	*2640*
8		*E-coli*	*2765*
9		*klebsilla*	*2707*
10		*pseudomonas*	*2200*

4.9.2Anti fungal activity

4.9.2.1. Procedure

4.9.2.2. Preparation of inoculums

The inoculums for the experiment were prepared in fresh sabouraud's broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards. The turbidity of the culture may be adjusted by the addition of sterile saline or broth (if excessive0 or by further incubating to get required turbidity.

4.9.2.3. Preparation of sterile swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or dry heat (only for wooden swabs) by packing the swabs in culture tubes, papers or tins etc.

4.9.2.4. Sterilization of forceps

Sterilize forceps by dipping in alcohol and burning of the alcohol

4.9.2.5. Experiment

The standardized inoculums is inoculated in the plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing by pressing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60 º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. Each Petri dish is divided into 4 parts, in each part samples disc such as AESP, EESP, and MESP (100µg) discs (discs are soaked overnight in sample solution) and Std Clotrimazole 10µg, are placed in 4^{th} part of the plate with the help of sterile forceps. Then Petri dishes are placed in the refrigerator at 4º C or at room temperature for 1 hour for diffusion. Incubate at 28 º C for 48hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.[59]

Table 4:Fungal strain used for the study with NCIM

Sl no	Fungi strains used	NCIM
1	*A.fumigatus*	1811
2	*M.purpureas*	1090
3	*A. paraticus*	2796
4	*Candida albicans*	3100
5	*A.niger*	1344

4.10. PHARMACOLOGICAL STUDY

4.10.1. Experimental animals

Female nulliparous, non pregnant Swiss albino mice (25- 30g) were used used for acute toxicity study. Mice with either sex used for the evaluvation of analgesic activity of methanolic extract of *sphenodesme paniculata*. Wister rats of either sex, weighing 180-220g used for the evaluation of anti inflammatory activity of methanolic extract of *Sphenodesme paniculata*. All animals were obtained from the animal house, KMCH College of Pharmacy, Coimbatore, Tamil Nadu. They were allowed food and water *ad libitum* up to the experimentation period. Prior to use, the mice were housed in polypropylene cages in group of six animals under standard environmental conditions (20-25^{0}C), natural light-dark (12h : 12h) cycle. Each animal was used only once under standard laboratory conditions. All the observations were made at room temperature in a noiseless diffusely illuminated room and were made between 9.00 to 17.00 h in the experimental room. All the experimental protocols were approved by Institutional Animals ethical committee (IAEC) of KMCH College of Pharmacy, Coimbatore–48

(KMCRET/M.Pharm/06/2014-15).

4.10.2 ACUTE TOXICITY STUDY

Acute oral toxicity refers to those adverse effects occurring following oral administration of a single dose of a substance or multiple doses given within 24 hours.

Table:5 Experimental protocol

NAME OF THE STUDY	**ACUTE TOXICITY**
Guideline followed	OECD 423 method – Acute toxic class method.
Animals	Healthy young Swiss albino female nulliparous non pregnant mice.
Body weight	25-30 g
Administration of doses	5,50,300, and 2000 mg/kg body weight
Number of administration	3 animals each in 4 groups
Route of administration	Oral
Room temperature	22ºC±3ºC
Humidity	45-55%
Light	12h:12h (light:dark cycle)
Feed	Standard laboratory animal food pellets with water *ad libitum*

4.10.2.1. Study procedure

Acute oral toxicity was performed as per Organization for Economic Co-operation and Development (OECD) guideline 423. The methanolic extract *of sphenodesme paniculata* was administered in a doses 5, 50, 300, and 2000 mg/kg body weight by gavage using specially designed mice oral needle. Animals were kept overnight fasting prior to dosing (food was withheld but not water). After the administration of methanolic extract of *sphenodesme paniculata*, food was withheld for 2h in mice. Animals were observed individually at least once during the first 30min, periodically during the first 24 h, with special attention given during the first 4 h, and daily thereafter for a total of 14 days .

4.10.3. SCREENING OF ANTI INFLAMATORY ACTIVITY

Inflammation is a tissue response to infection, injury, irritation, foreign substance. It is a part of host defense mechanism but when it becomes uncontrolled it is a hopeless condition. Several tissue factors involved in inflammatory reaction such as release of histamine, bradikinin and prostaglandins. The inflammatory reaction is readily produced in rats in the form of paw oedema with help of irritants such as carrageenan, histamine, serotonin ,bradikinin, formalin, etc.[60]

4.10.3.1. Experimental design

Wistar albino rats weighed around 180-220were used for this study .They were randomly devided into 4 groups of 6 rats in each, in carrageenan induced inflammatory model .Diclofenac 50mg/kg used as a standard drug and indomethacin 10 mg/kg used as a standard in carrageenan induced inflammattory model and histamine induced inflammatory model respectively

Group-1: Served as control which received carrageenan only

Group-II: Served as positive group which received Diclofenac (50mg/kg.p.o) in carrageenan induced inflammattory model and indomethacin(10mg/kg p.o) in histamine induced inflammattory model

Group-III:Served as test group which received methanolic of *methanolic extract of sphenodesme paniculata* (200mg/kg,p.o) and carrageenan

Group-IV:Served as a test group which received of *methanolic extract of sphenodesme paniculata* (400mg/kg,p.o) and carrageenan.[61]

4.10.3.1.1.Anti-inflamatory activity by carrageenan induced paw oedema in rats

In this experiment, carragenan induced rat hind paw edema was used as acute inflammation according to winter. Adminstration of carrageenan in the sub planar region of rat's hind paw leading to the formation of edema in situ due to the localized inflammation. Wistar albino rats weighed around 180-220were used for this study. They were randomly divided into 4 groups of 6 rats in each. Group I control received 0.1 ml 1 % w/v solution of carrageenan into sub planar region of right hind paw of rats of each rats of every group. Group 11 positive control

received 50 mg/kg body weight of diclofenac sodium orally. Group III and 1V received methanolic crude extract 200mg /kg and 400mg /kg respectively.. The paw volume was measured by the plethysmometer(Ugo Basile,7140,Italy)at 1,2,3,4,5 and 6 h after carrageenan injection.[62,68]

4.10.3.2. Histamine induced rat paw edema

Inflammation was induced by injection of 0.1 ml of freshly prepared histamine (1%)aqueous suspension in normal saline underneath the plantar tissue of the right hindpaw of rats .The drug treatment and paw volume was measured in a similar manner to that of carrageenan induced paw edema model. 1 hr after administration of standard drug indomethacin (10 mg/kg, i.p.).. The paw volume was measured by the plethysmometer(Ugo Basile,7140,Italy) at 90 min and 180 min after histamine injection.[63]

4.10.4. Analgesic activity

Analgesia is defined as state of reduced awareness to pain and analgesics are the substance which decreases pain sensation by increasing threshold to pain stimuli. Commonly used analgesics are aspirin, paracetamol and morphine.

Pain reaction in experimental animals can be produced by applying noxious stimulus such as thermal, chemical and physical pressure[64].

4.10.4.1. Experimental design

Swiss albino mice weighed around 20-25g were used for this study .They were randomly devided into 4 groups consisting of 5 mice each. Morphine sulphate (5mg/kg.s.c) used as a standard in Eddy's hot plat method and diclofenac(50mg/kg. p.o) used as a standard in acetic acid induced writhing model.[58]

Group-I: Served as control which received vehicle only

Group-II: Served as positive group which received morphine sulphate (5mg/kg.s.c) for hot plat analgesic model and diclofenac (50mg/kg. p.o) for acetic acid induced writhing model

Group-III: Served as test group which received methanolic extract of *sphenodesme paniculata* (200mg/kg,p.o)

Group-IV:Served as a test group which received methanolic extract of *Sphenodesme paniculata* (400mg/kg,p.o)[65]

4.10.4.1.2.Hot Plate Test

Mice of both sexes were screened based on their response when subjected to hot-plate. Test groups and control received their respective treatments at the proper doses as mentioned earlier, while the positive control group received morphine (5 mg/kg, i.p.). Pain stimulus was produced by placing the animals on hotplate maintained at the temperature of 55 ± 0.5$^{\circ C}$. Paw licking or jumping off the plate was considered as response to pain stimulus. Reaction time for each group was recorded at 0, 30, 60 and 90 during the observation period. To avoid any possible accidental paw damage, a cut-off point of 15 sec was considered .Reaction time of the extract and standard was compared with the control.[66]

4.10.4.3. Acetic acid induced writhing

Analgesic activity of methanolic extract of *sphenodesme paniculata* was evaluated by acetic acid induced writhing method . Test groups and control received respective treatments at the proper doses, while the positive control group received diclofenac sodium(50 mg/kg, p.o.). Thirty minutes later, acetic acid (0.7%,10 ml/kg, i.p.) was administered to each mouse to induce abdominal contraction known as writhing. After an interval of 5 min, number of writhes for each group was counted for 10 min and recorded. The number of writhes of test groups at different dose levels, and standard were compared with the control.[67]

4.11.STATISTICAL ANALYSIS

The data's of all the parameters were analyzed using the software Graph pad Prism 5. Analysis of variance (ANOVA); one way ANOVA followed by Dunnet's test was performed. The values were expressed as Mean ± SEM.

5. RESULTS AND DISCUSSION

5.1. Percentage yield of the different crude extracts of *Sphenodesme paniculata*(Clarke)

The yield of crude ethyl acetate extract, methanolic extract and aqueous extract of*Sphenodesme paniculata* was found to be: 11.2 ± 0.09%w/w,19.0 ±0.45%w/w and 12.75 ± 0.88%w/w respectively.

5.2.Phytochemical analysis

All the extracts were subjected to preliminary phytochemical analysis for detecting the presence of alkaloid, flavanoids, phenolic tannins, glycoside, steroids and carbohydrate. The presence of different phytoconstituents in ethyl acetate, methanolic and aqueous extracts of *Sphenodesme paniculata* were detected indifferent solvents as part of preliminary phytochemical investigation

Table 6: Qualitative analysis for phytoconstituents

Phytoconstituents	Ethyl acetate extract	methanolic extracts	Aqueous extract
Alkaloid	+++	+++	-
Glycoside	++	+	++
Flavanoids	+++	+	++
Phenolic & Tannins	-	+	+
Saponins	-	-	-
Steroid	-	+++	-
Carbohydrate	-	-	+

-ve = absent +ve = Present, ++ = More present, +++ = High amount

5.4. Total phenolic contents

Polyphenols are the largest group of phytochemicals that are gaining acceptance as being responsible for the health benefits. Because of their chemical structure, plant polyphenols can scavenge free radicals and inactive other pro-oxidants, and also interact with a number of biological relevance. Total phenol content (TPC) for theethyl acetate extract, methanolic extract and aqueous extract of*Sphenodesme paniculata* were determined by Folin-ciocalteu's method and the TPC of the eachextract were presented in Table 2.

Table 7: Data of concentration and absorbance of standard gallic acid

Sl. No	**sample**	**Concentration** (µg/ml)	Absorbance (725nm)
1	Gallic acid	**10**	**0.072**±0.12%
		20	**0.15**±0.32%
		40	**0.29**±0.06%
		60	**0.37**±0.14%
		80	**0.53**±0.19%
		100	**0.68**±0.41%
2	Ethyl acetate extract	**1000**	**0.31**±0.26%
3	Methanolic extract	**1000**	**0.48**±0.30%
4	Aqueousextract	**1000**	0.26±0.16%

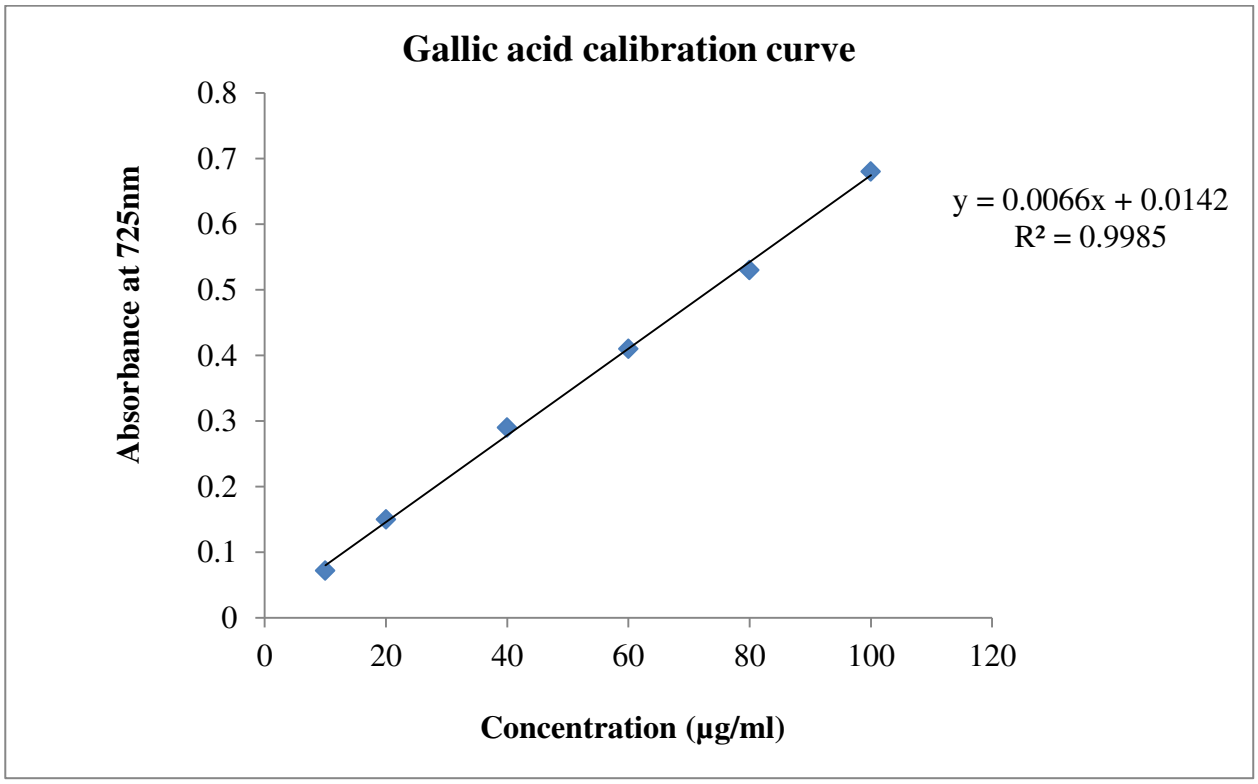


Fig. 5: Calibration curve of standard gallic acid for total phenolic contents

Table 8: Total phenolic content of extracts

Sl. No	Extracts	Total phenolic content in mg GAE/g
1	Ethyl acetateextract	41.4±0.02
2	Methanolic extract	68.6±0.02
3	Aqueousextract	36.1±0.01

The TPC was found to be high in methanolic extracts when compared to ethyl acetate and aqueous extracts. The Table 2 reveals that the methanolic extract contains high amount of total phenols which was about 68.6mg GAE/g for the MESP, 41.4mg GAE/g for the EESP and 36.1mg GAE/g for AESP

5.5. Totalflavanoids contents by UV spectrophotometer

The total flavanoids content was determined by aluminum chloride colorimetric method. The principle of aluminum chloride colorimetric method for determination of flavanoids is based on the fact that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonol. In addition, aluminum chloride forms acid labile complexes with the orthodihyroxyl groups in the A or B ring of flavanoids. Flavonoid with C-3 and C-5 hydroxyl groups, such as quercetin form complexes with aluminum chloride, showing maximum absorbance at 415 nm. Generally the aluminum chloride complexes of compounds with more functional groups absorb more strongly at 415nm and show the absorption maximum at longer wavelength.

Table 9: Data of concentration and absorbance of standard quercetin

Sl. No	**sample**	**Concentration** (µg/ml)	Absorbance (415nm)
1	Quercetin	**10**	**0.079** ± 0.01
		20	**0.241** ± 0.03
		40	**0.596** ± 0.05
		60	**0.99** ± 0.01
		80	**1.50** ± 0.05
		100	**2.022** ± 0.01
2	Ethyl acetateextract	**1000**	**0.281**± 0.02
3	Methanolic extract	**1000**	**0.434**± 0.07
4	Aqueousextract	**1000**	**0.21**± 0.08

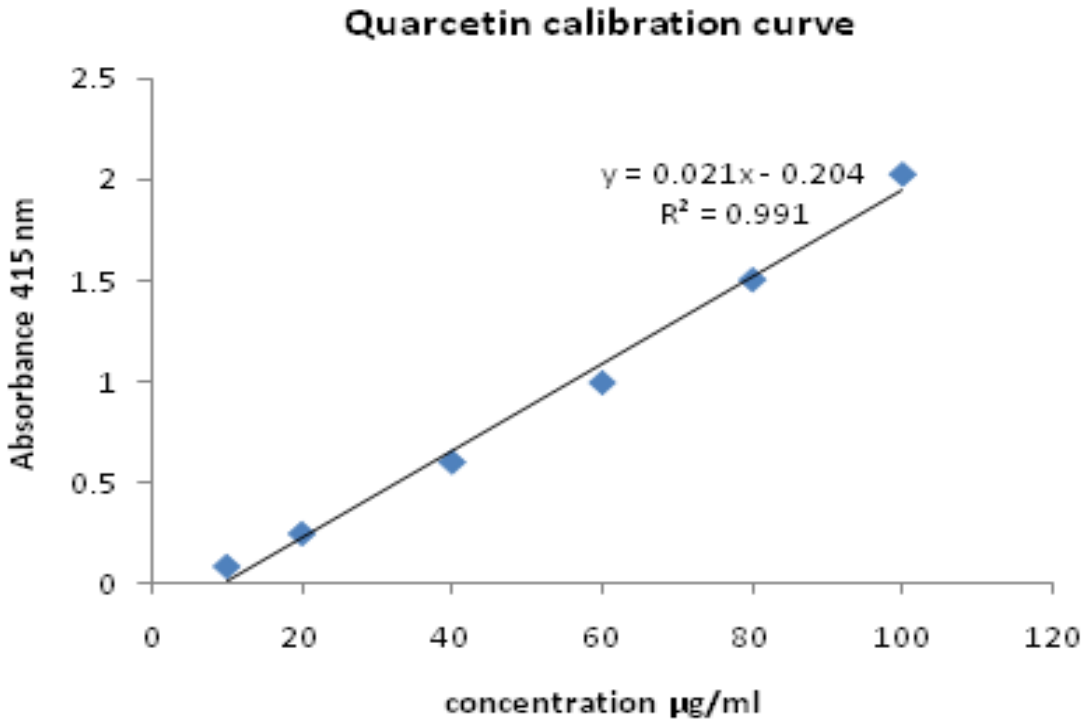


Fig .6:Calibration curve of standard quercetin for total flavanoids contents

Table 10: Total flavanoids content of extracts

Sl. No.	Sample	Total flavanoids content in mg QE/g
1	Ethyl acetateextract	21.61±0.02
2	Methanolic extract	28.47±0.01
3	Aqueousextract	18.29±0.01

Total flavanoid content of the extracts were determined and presented in Table 4.It reveals that the methanolic extract exhibited high flavanoids content when compared to ethyal acetate extracts and aqueous extract which was about 28..47mg GAE/g for the MESP, 21.61mg GAE/g for the EESP and 18.2mg GAE/g for AESP

5.6.3. Total antioxidant activity

Antioxidants have been found to exhibit their activity by various mechanisms at different stages of oxidation reaction. Phosphomolybdenum assay, initially developed by (Prieto et al., 1999), was used for the evaluation of total antioxidant capacity of extracts and the values are presented in Table18. The assay was based on the reduction of Mo (IV) to Mo (V) by the sample and subsequent formation of a green phosphate Mo (V) complex at acidic PH.

Table 11: Estimation of total antioxidant capacity of methanolicextract of*Sphenodesme paniculata*

Sl No	sample	Concentration µg/ml	Absorbance 695nm
1	Ascorbic acid	100	0.211±0.31%
		200	0.54±0.21%
		300	0.73±0.43%
		400	1.05±0.19%
		500	1.45±0.32%
		600	1.8±0.04%
2	Ethyl acetate extract	1000	0.61±0.06%
3	Methanolic extract	1000	0.89±0.35%
4	Aqueousextract	1000	0.29±0.41%

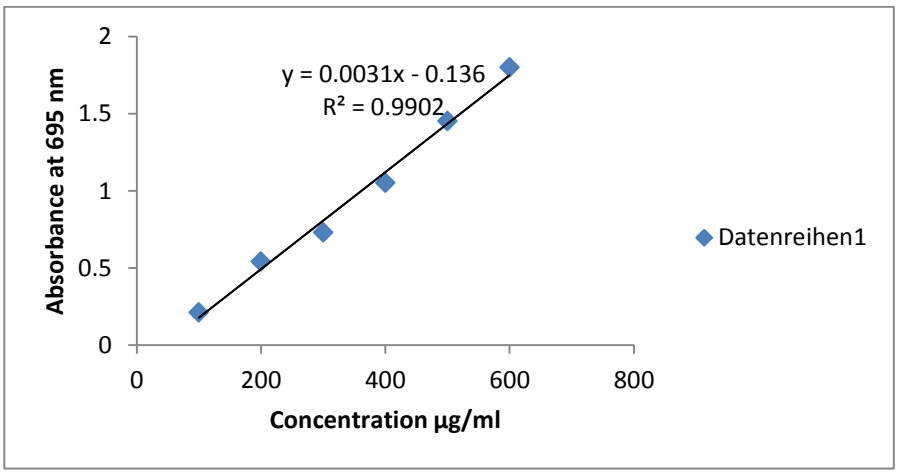


Figure. 07: Estimation of total antioxidant capacity of ofmethanolicextract of *Sphenodesme paniculata*

Table 12: Total antioxidant capacity of plant extracts

Sl. No.	Sample	Total antioxidant capacity (AAE µg/g)
1	Ethyl acetate extract	301.6±0.05
2	Methanolic extract	342.8±0.02
3	Aqueousextract	137.3±0.03

There was a difference in TAC between ethylacetat extract,methanolicand aqueous extrcts were observed and the methanolicextracts exhibited more activity than ethyl acetate extract, which was about 342.8 mgAAE/g for the MESP, 301.6g GmgAAE/g for the EESP and 137.3mg AAE/g for AESP.

5.3. HPTLC finger print profile of methanolic extracts of *Sphenodesme paniculata*

3.5.2.1. Development of optimum mobile phase

Chromatographic separation studies were carried out on the working standard solution of (1mg/ml) in methanol. Initially, various trials were carried out with different solvent systems. Toluene: ethyl acetate: formic acid: methanol ((3:6:1.6:0.4**v/v**) was selected for simultaneous determination of rutin, quercetin, gallic acid showing a sharp and well-defined peak. Well-defined bands were obtained when the chamber was saturated with the mobile phase for 30 min at room temperature. The standard band of gallic acid (R_f= 0.47), quercetin (R_f= 0.67)andrutin (R_f= 0.08) along with alcoholic extracts of *Sphenodesme paniculata* separated on HPTLC plate was scanned at 254 nm (Fig. 8 and 9).

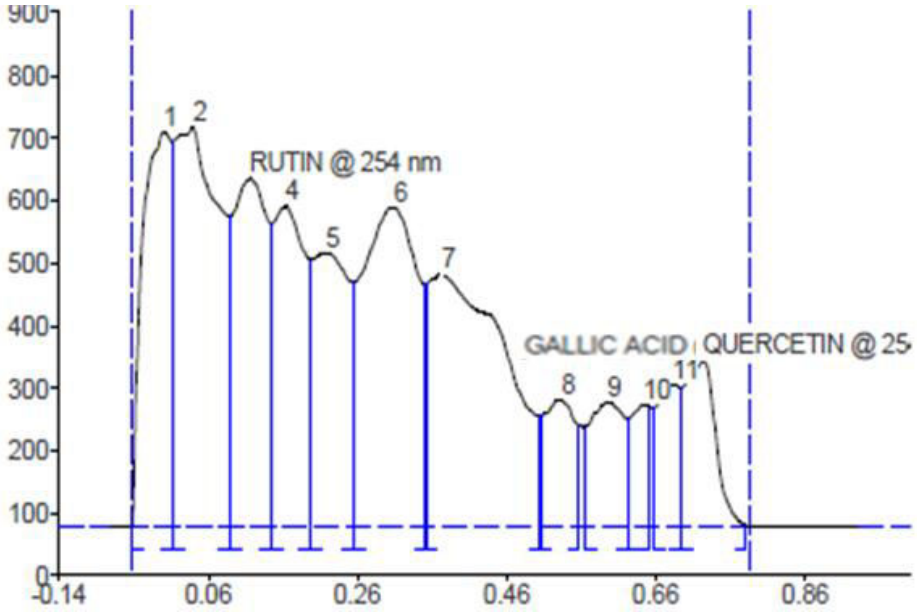


Fig.08. Simultaneous HPTLC densitogram of methanolicextracts of *Sphenodesme paniculata*showing separation ofpolyphenolsin the solvent system: toluene: ethyl acetate:formic acid: methanol (3:6:1.6:0.4**v/v**) **at 254 nm.**

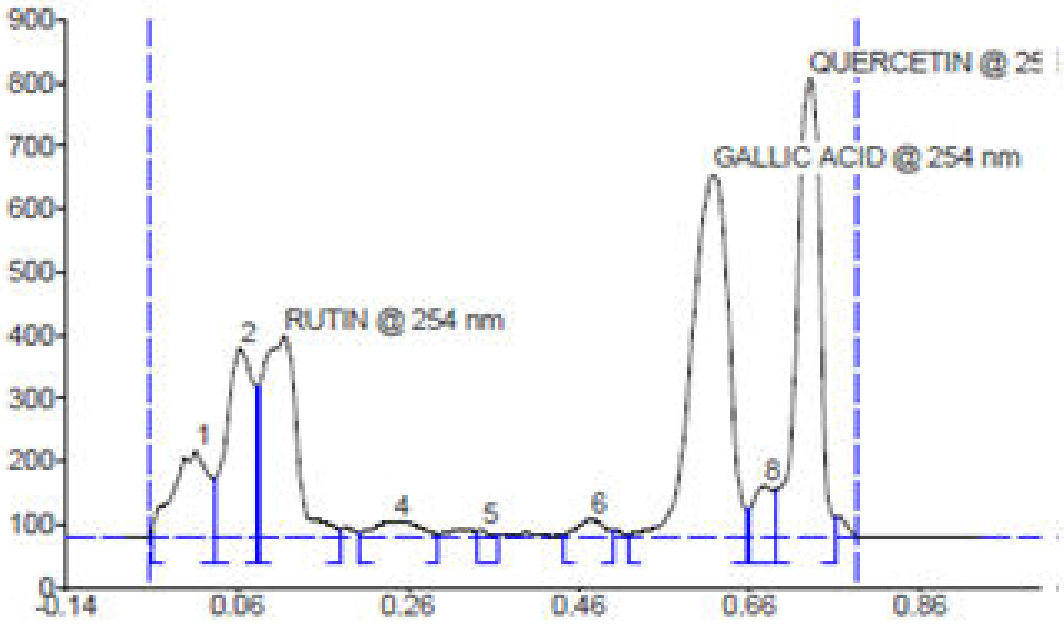


Fig.09.

Simultaneous HPTLC densitogram of polyphenols in the solvent system: toluene: ethyl acetate:formic acid: methanol (3:3:0.8:0.2 v/v) at 254 nm.

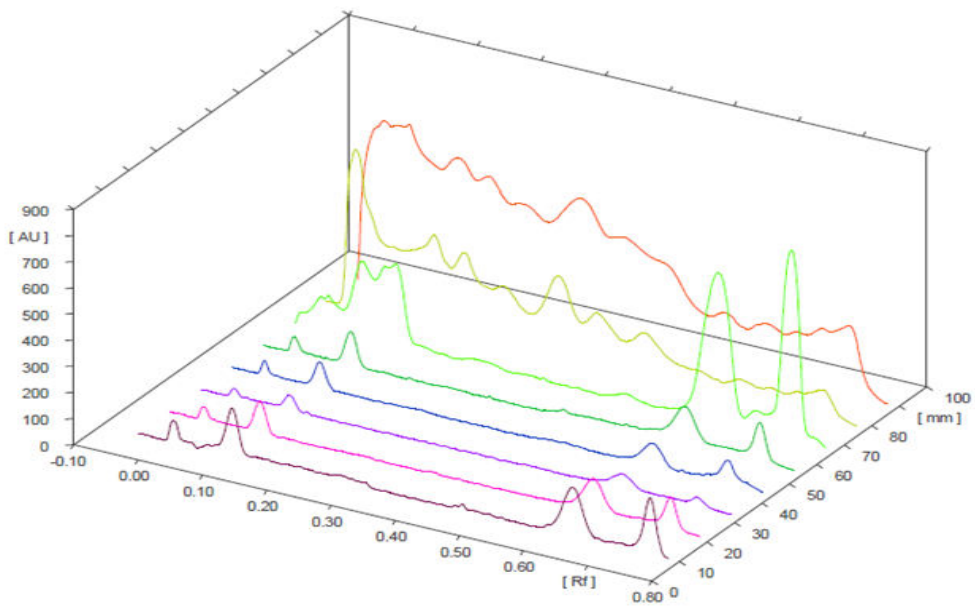


Fig. 10: 3D display of all tracks of methanolic extract of *Sphenodesme paniculata* and markers of at 254 nm

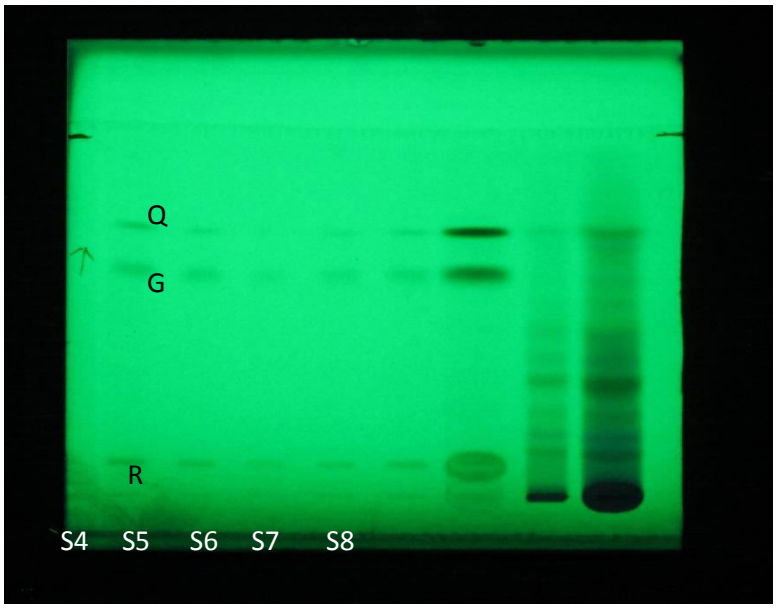


Figure 11:detection of band of HPTLC

Sample 1: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 5.0 µl

Sample 2: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 7.0 µl

Sample 3: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 9.0 µl

Sample 4: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 11 µl

Sample 5: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 13 µl

Sample 6: Gallic acid (G), Quercetin (Q), Rutin (R) (STD) 15 µl

Sample 7: MESP 10 µl

Sample 8:MESP 15 µl

Number of spots were developed when extract of *Sphenodesme paniculata* was runned on HPTLC, two spots coinsides with the RF values of Quercetin (Q) and Rutin (R)

5.6. *In vitro* antioxidant studies

5.6.1 DPPH radical scavenging activity

DPPH radical scavenging assay is widely employed and preferred for the measurement of antioxidant activity. DPPH in methanol occurs as free radical and showed pink colored solution which becomes faint after acquiring proton from the antioxidant, DPPH becomes a diamagnetic stable molecules. Hence, measurement of reduction in the color intensity of methanolic DPPH solution may be used to evaluate the antioxidants strength to donate proton. This accepted odd electron plays the role of a free radical scavenging antioxidant and gives a strong absorbance which is maximum at 517nm.

DPPH radical scavenging ability ofmethanolic extracts of *Sphenodesme paniculata*waspresented in Table 8 & 9. The IC50 values formethanolic extracts of *Sphenodesme paniculata* was 6.49mg/ml. The DPPH radical scavenging ability of the extracts was less than that of ascorbic acid. The study showed that the extract have moderate hydrogen donating ability.

Table 13: % inhibition and IC50 values of DPPH radical by ascorbic acid

Sl. No	Concentration (µg/ml)	% inhibition	IC50 (µg/ml)
1	**0.5**	28.1±0.15%	**2.63**
2	**1**	**43.9**±0.11%	
3	**2**	**56.8**±0.01%	
4	**3**	**61.3**±0.06%	
5	**4**	**83.9**±0.23%	
6	**5**	**90.1**±0.09%	

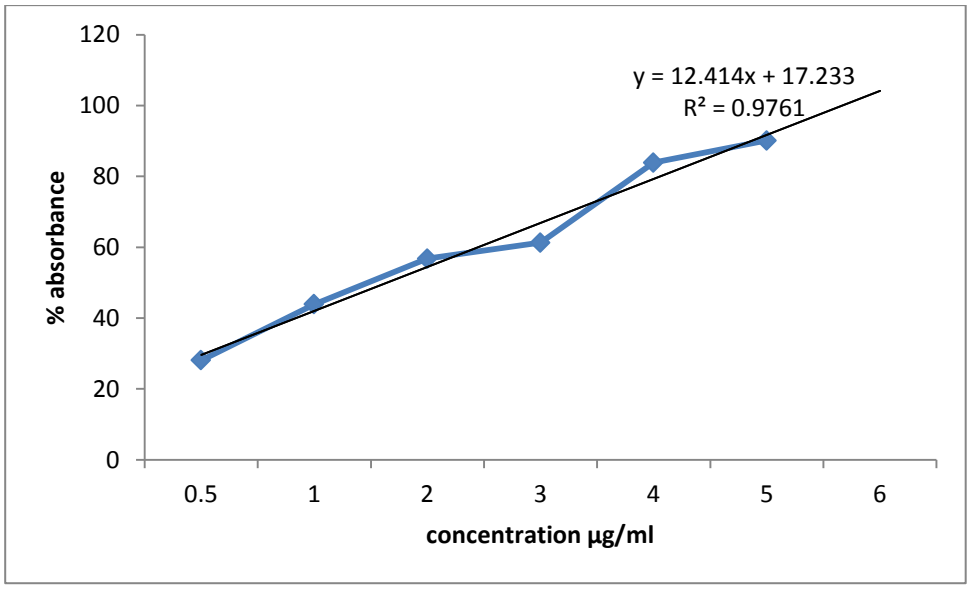


Figure 12: DPPH radical scavenging activity of Ascorbic acid

Table 14: % inhibition and IC50 values of DPPH radical bymethanolic extract of *Sphenodesme paniculata*

Sl. No	Concentration (µg/ml)	% inhibition	IC50 (µg/ml)
1	**1**	**30.2**±0.23%	**6.49**
2	**2**	**35.7**±0.15%	
3	**4**	**38.3**±0.11%	
4	**6**	**41.6**±0.35%	
5	**8**	**44.1**±0.41%	
6	**10**	**48.5**±0.18%	

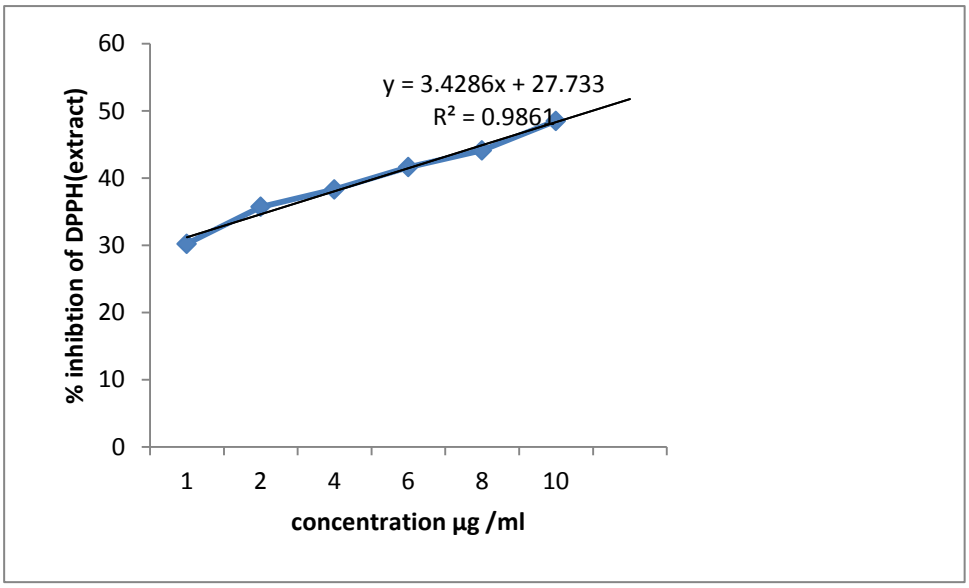


Figure 13: DPPH radical scavenging activity of methanolic extract of *Sphenodesme paniculata*

5.6.2. Total antioxidant activity by abts radical cation assay

ABTS radical scavenging activity is used for screening complex antioxidant mixtures such as plant extracts. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decrease with the scavenging of the proton radicals.

ABTS radical scavenging ability of extracts were presented in Table15& 16. The IC50 values 0f extract was 6.1µg/ml., whereas the IC50 value for the standard ascorbic acid was found to be 4.11µg/ml. The present study reveals that the extracts were posseshigher antioxidant capacity when compared to standard ascorbic acid.

Table 15: % inhibition of ABTS radical by Ascorbic acid

Sl. No	Concentration (µg/ml)	% inhibition	IC50 (µg/ml)
1	**0.5**	**17.1**±0.13%	**4.11**
2	**1**	**26.3**±0.0 3%	
3	**2**	**39.1**±0.09%	
4	**3**	**46.9**±0.21%	
5	**4**	**48.7**±0.36%	
6	**5**	**69.1**±0.18%	

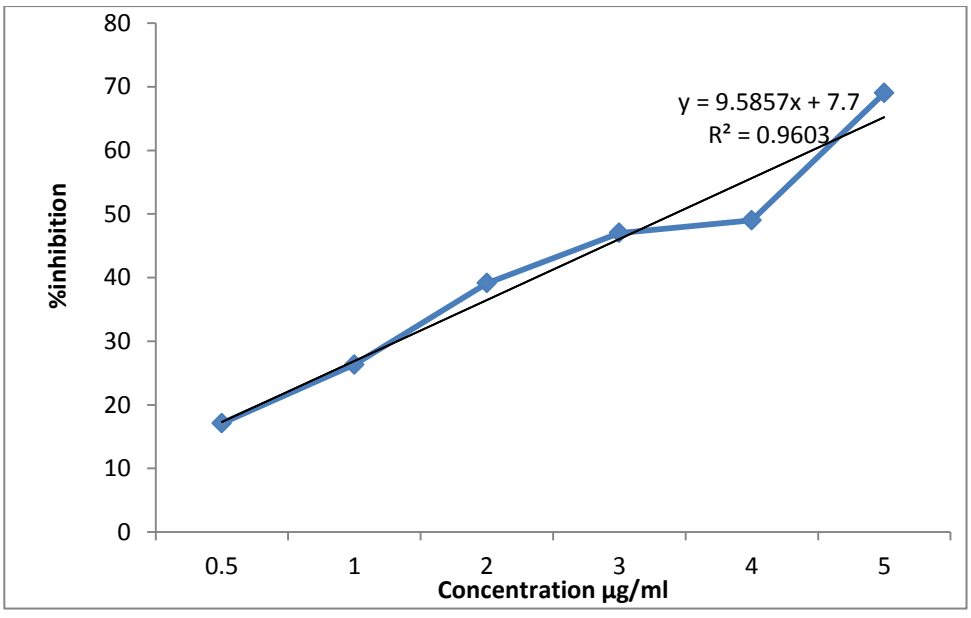


FIG14 : ABTS radical scavenging activity of Ascorbic acid

Table 16: % inhibition and IC50 values of ABTS radical bymethanolic extract of *Sphenodesme paniculata*

Sl. No	Concentration (µg/ml)	% inhibition	IC50 (µg/ml)
1	1	**15.1**±0.13%	6.1
2	2	**26.7**±0.0 3%	
3	4	**29.1**±0.09%	
4	6	**38.9**±0.21%	
5	8	**44.7**±0.36%	
6	10	**52.1**±0.18%	

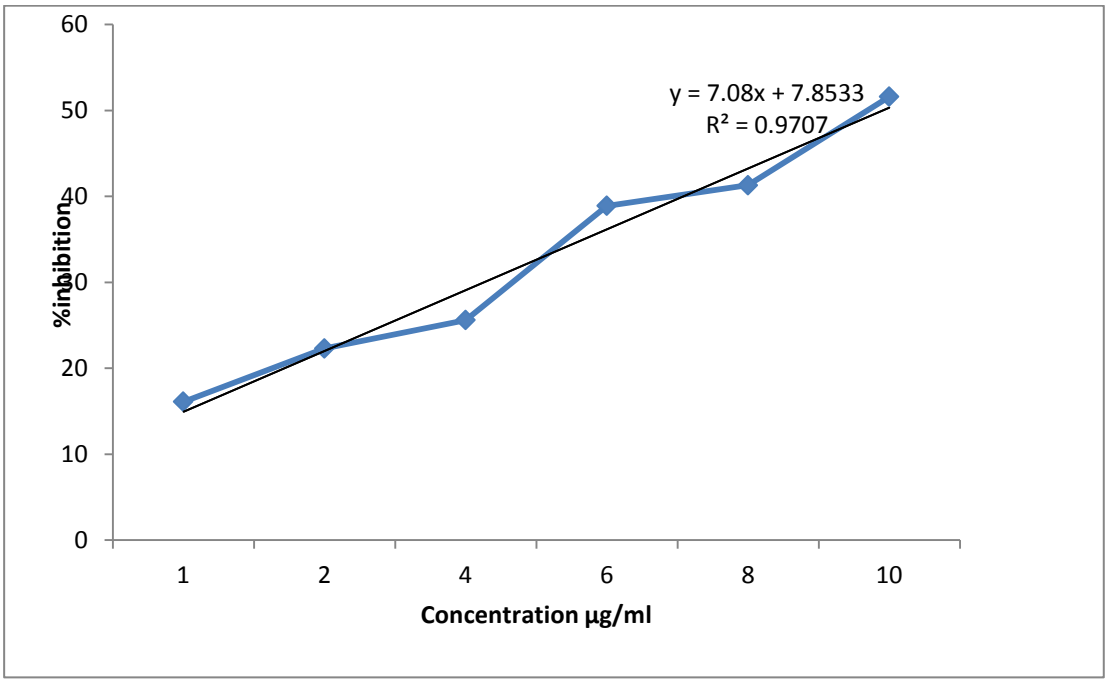


Figure 15: ABTS radical scavenging activity of methanolic extract of *Sphenodesme paniculata*

5.7. Cytotoxicity assay of methanolic, ethyl acetate and aqueousextractsinhela cells

Based on the MTT assay, the non-cytotoxic concentration of the extracts was determined. The cytotoxicity experiment showed that all tested extracts were non-cytotoxic to HeLa cells up to a concentration of 200 µg/ml, as shown in Table 23

Table 17: % Cell viability of various concentrations of leaf extracts

SI NO	Concentration (µg/ml)	%Cell viability		
		Ethyl acetate	Methanolic	Aqueous
1	12.5	100.4082	100.5102	100.6122
2	25	97.55102	100.2041	100.3061
3	50	84.59184	96.42857	91.02041
4	100	55.61224	91.93878	85.40816
5	200	23.26531	89.38776	74.79592

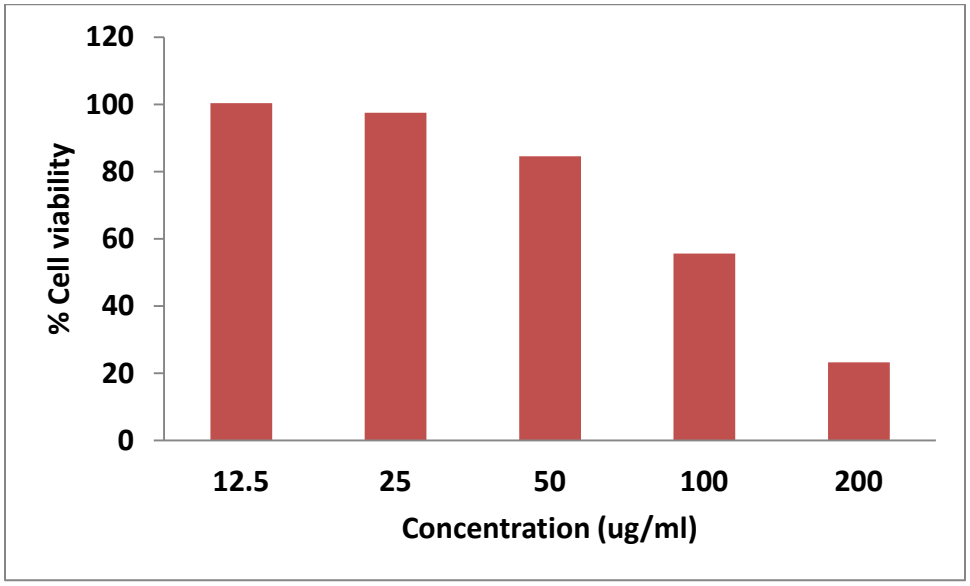


Fig. 16. Cytotoxicity assay of ethyl acetate extracts on cytotoxicity in HeLacells. Cells in 96 well plates (1×10^6 cells/ well) were incubated with different concentrations of crude extracts (12.5- 200µg/ml) for 48 h.

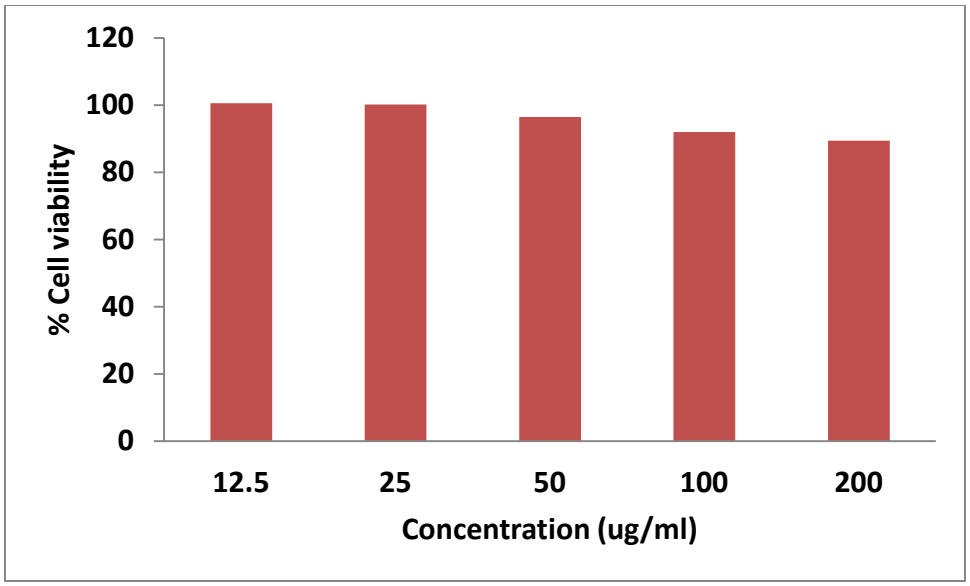


Fig. 17. Effect of methanolic extract on cytotoxicity in HeLacells. Cells in 96 well plates (1×10^6 cells/ well) were incubated with different concentrations of crude extracts (12.5-200µg/ml) for 48 h.

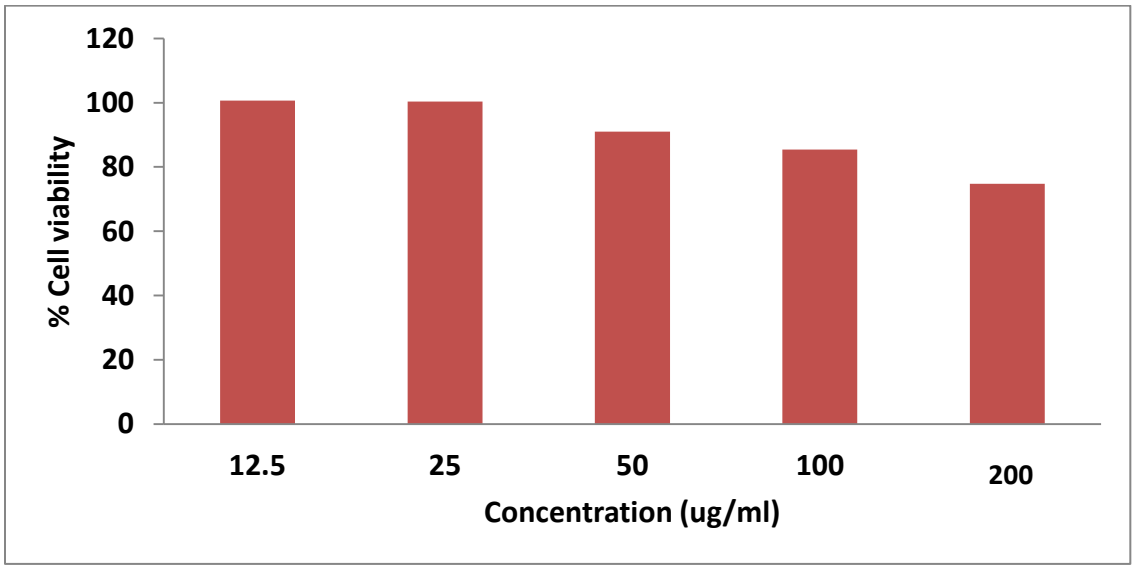


Fig. 18. Cytotoxicity assay of aqueous extracts on cytotoxicity in HeLacells. Cells in 96 well plates (1×10^6 cells/ well) were incubated with different concentrations of crude extracts (12.5- 200µg/ml) for 48 h.

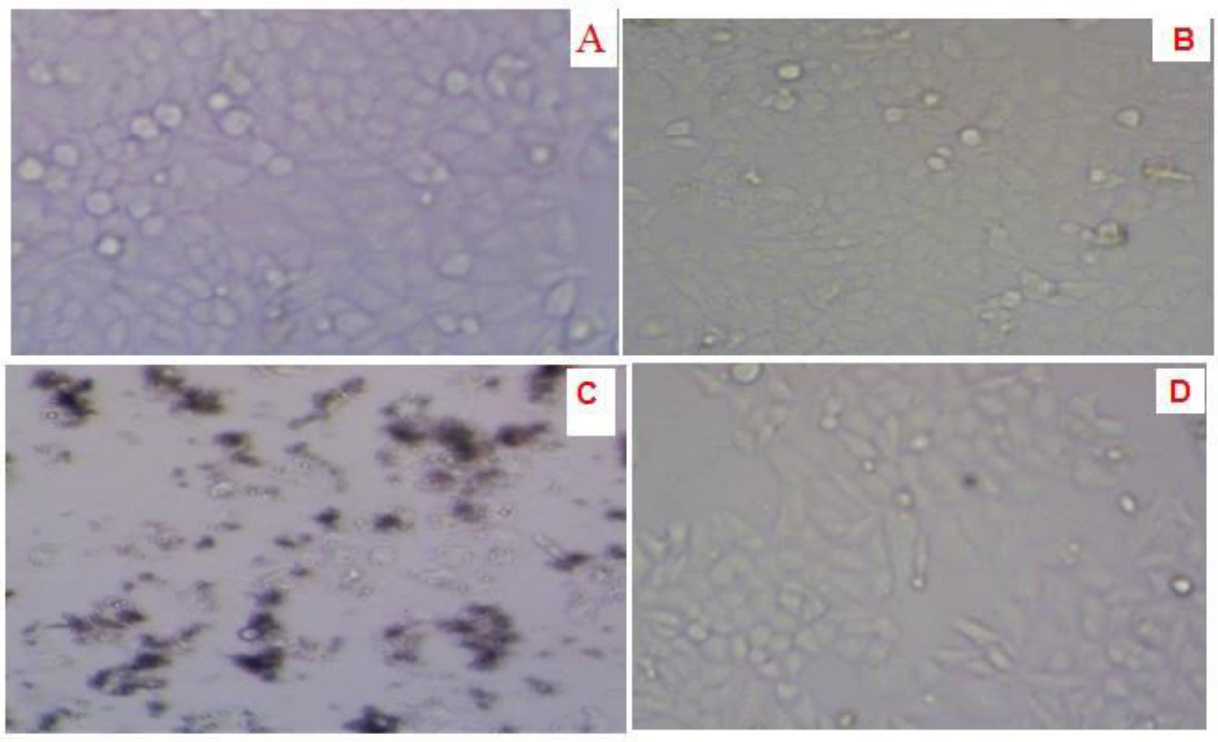


Figure 19: Photomicrograph (10X) of HeLa cells lines treated extracts A-control , B-aqueous extract(200 µg/ml), C-ethyl acetate extract(200 µg/ml), D-methanolic extract(200 µg/ml)of*Sphenodesme paniculata*for 48 h.

5.8. Antimicrobial activity of stem of *Sphenodesme paniculata*

The anti microbial study was carried out for ethyl acetate ,methanolic and aqoues extract of Sphenodesme paniculata against different strain of bacteria (5 Gram + ve and 5 G – ve) and fungai, by the disc diffusion method at 200µg/disc. The ciprofloxacin 10µg/disc and fluconazole 10µg/disc were used as standard for bacteria and fungai respectively

The standard ciprofloxacin and fluconazole was found to have significant antimicrobial activity against bacteria and fungi respectively.The various zones of inhibition was observed from all the extracts against various strain .Among the extracts themethanolic extract of *Sphenodesme paniculata* was observed to have significant antimicrobial activity than that of ethyl acetate and aqueous extracts.The zone of inhibition was observed from both Gram +ve and Gram –

vebacteria and fungal strains .The maximum zone of inhibition was found in methanolic extract of *Sphenodesme paniculata*against*B.subtilis and Pseudomonasaeroginosa.*

5.8.1.Anti bacterialactivityactivity of stem of *Sphenodesme paniculata*

Table .18

Name of extract	Diameter of zone of inhibition in mm									
	Gram +ve strain					Gram –ve strain				
	S.**lentus**	s.*albus*	S.*aureus*	*B.subtilis*	B.*lentus*	*V.cholerae*	Coryne bacterium	*E-coli*	*klebsilla*	Pseudomonas *aeroginosa.*
STD	30	15	13	13	21	32	28	18	15	16
AESP	13	12	12	10	7	9	11	11	10	10
EESP	12	12	10	11	10	10	12	11	12	12
MESP	14	13	12	16	12	13	14	12	13	15

STD=Ciprofloxacin

5.8.2Anti fungal activityactivity of stem of *Sphenodesme paniculata*

All the extract of *Sphenodesme paniculata* showedanti fungalactivity.Theanti fungal activity was observed maximum in methanolic extract against *A.fumigates,*M.purpureusandC.albicans(zone of inhibition,12mm,12mm and 10mm) in table:19

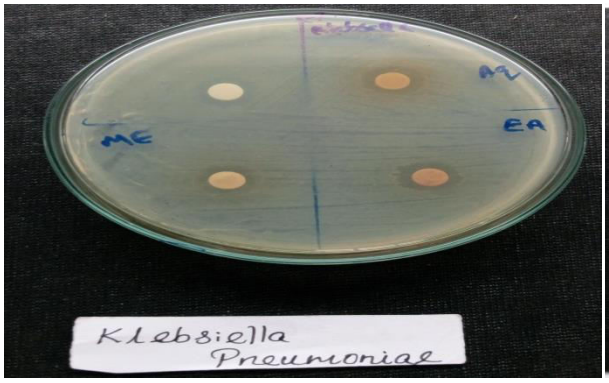
Table 19 :Anti fungal activityactivity of stem of *sphenodesme paniculata*

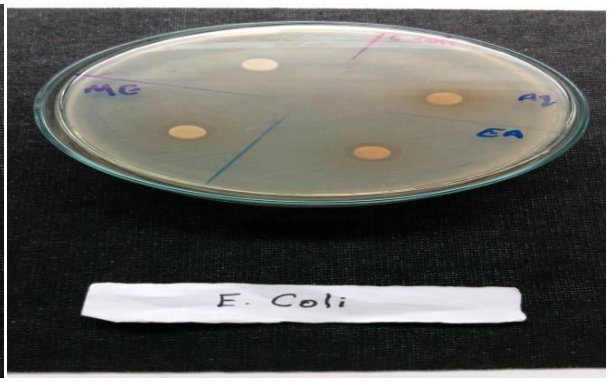
Name of extract		Diameter of zone of inhibition in mm			
	A.fumigates	*A.paratigus*	*M.purpureus*	*C. albicans*	*A.niger*
STD	30	15	28	17	28
AESP	10	6	11	11	9
EESP	9	9	10	9	10
MESP	12	10	12	11	10

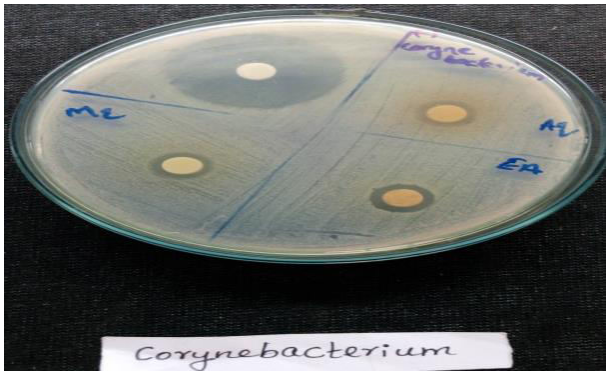
STD=Fluconazole

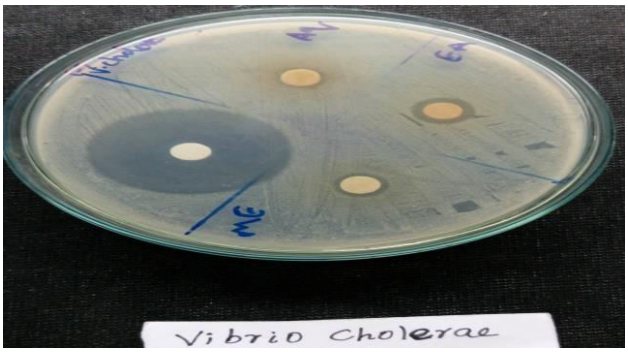
Anti bacterialactivit

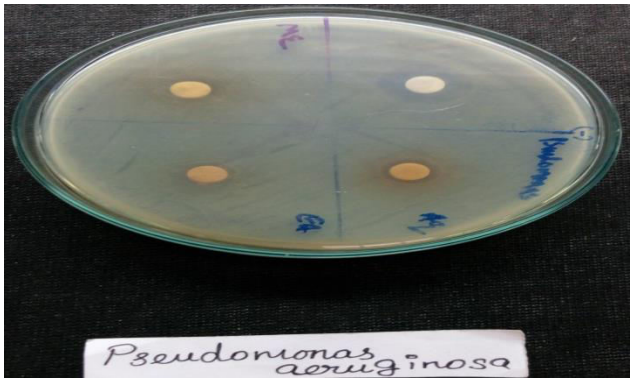
G- VE Bacteria



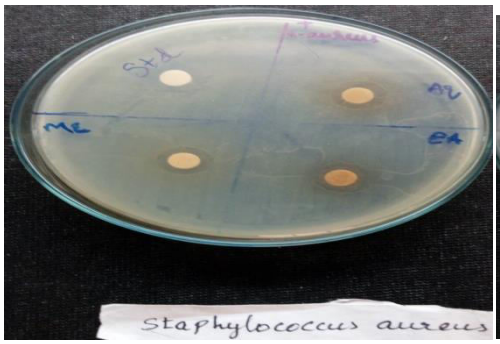


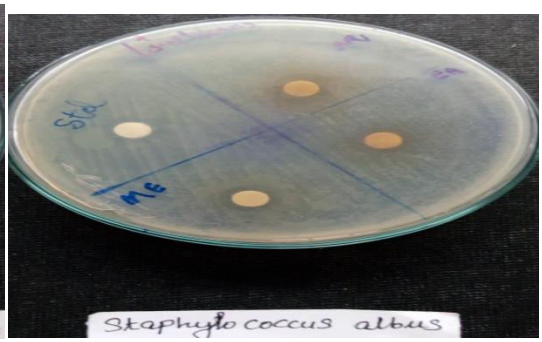


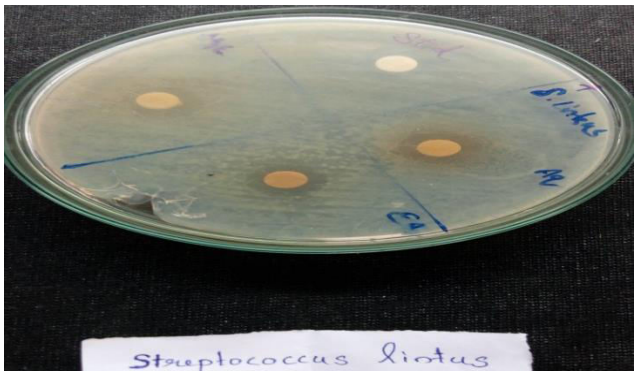


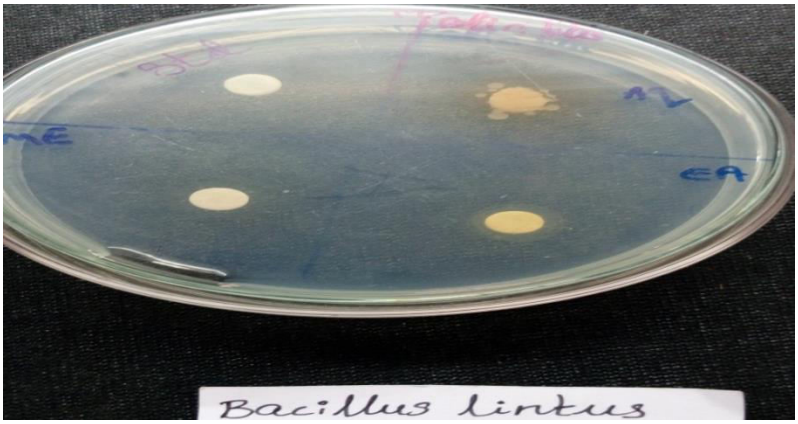


G -+VE Bacteria









Anti fungal activity

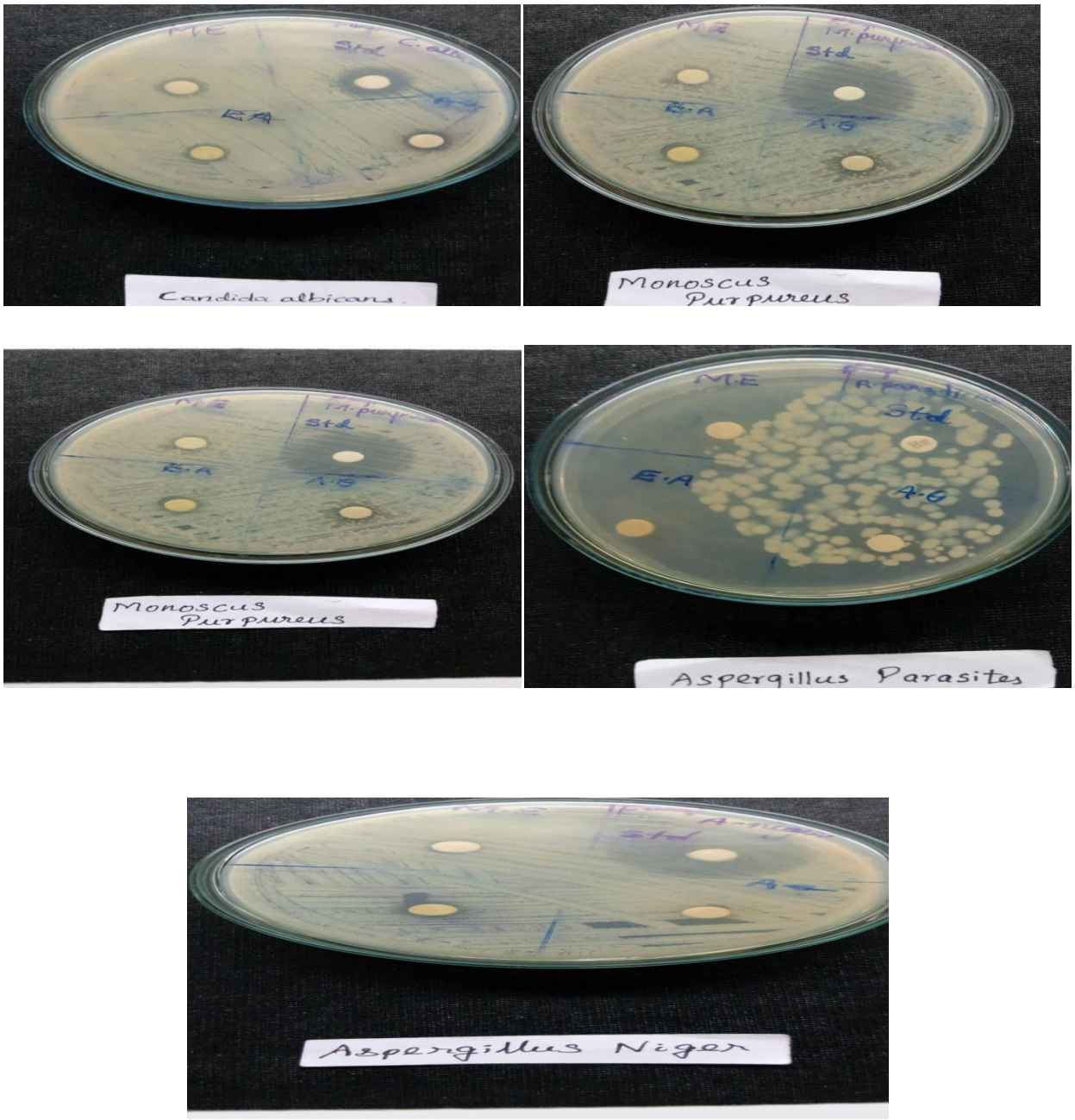


Figure 20: Photograph of anti microbial activity of *sphenodesmepanicula*

5.9. Screening of anti-inflammatory activity of methanolicexract of *Sphenodesme paniculata*

The Methanolic stem extract of *Sphenodesme paniculata*was subjected to screen the anti inflammatory activity by carrageenan and histamine induced models in the wistar albino rats.

5.9.1. Carrageenan-induced paw oedema model

The development of carrageenan induced paw edema is biphasic, the first phase occur within one hour of carrageenan administration and this attributed the release of cytoplasmic enzymes, histamine and serotonin from the mast cells. The second phase begin after one hour and remain through the 3 hr which is mediated by increased release of prostaglandins in the inflammatory area .hydrocortisone and some NSAIDS strongly inhibit the second phase of carrageenan induced edema but some others are effective against both phases.

The experimental rats were divided into four groups.The group-I received carrageenanonly. Group-II were administered with standard drug diclofenac (50mg/kg).Group-III and IV received test extracts at dose of 300 and 400 mg/kg respectively, After induction of carrageenan, the paw edema volume was measured for all the groups at an interval of 1 hour from the period of 1 to 6 hours

The oral administration methanolicexract at a doses 200 and 400mg /kg significantly suppress the paw edema at 2 to 6 hr after carrageenan injection in rats, at 6 hr high dose of exract showed 45% action and stranded showed 63% action ,so the extract is little anti histaminic and more effective in second phase. Diclofenac showed more activity in 2 to 6 hr

Table 20: Effect of methanolic extract on Carrageenan-induced paw oedema model

Groups	**Treatment and Dose**	Paw volume(ml) and % inhibition					
		1 hr	**2 hr**	**3 hr**	**4 hr**	**5 hr**	**6 hr**
I	Control	1.12±0.05	1.16±0.05	1.19±0.04	1.23±0.06	1.24±0.06	1.24±0.08
II	Diclofenac 50mg/kg	99.1±0.02 *	0.84±0.04 ***	0.81±0.03 ***	0.79±0.04 ***	0.77±0.03 ***	0.75±0.05 ***
III	MESP (200mg/kg)	1.05±0.03	0.97±0.04 *	0.99±0.08 *	1.02±0.03	1.09±0.02	1.09±0.08
IV	MESP(400 mg/kg)	1.02±0.03	0.87±0.03 **	0.85±0.04 **	0.86±0.05 **	0.92±0.08 **	0.97±0.06 **

Statistical comparison: Each group (n=5), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$, compared to control

5.9.2. Histamine induced rat paw edema model

The oral administration methanolic exract at a doses 200 and 400mg /kg significantly suppress the paw edema at 90 and 180 hr after histamine injection in rats paw. High dose of exract showed 51 % inhibition at 90 min and standard indomethacin 10 mg showed 58.3% inhibiton, so the extract showed anti histaminic activity al

Table 21: Effect of methanolic extract in histamine induced rat paw edema

Groups	Treatment and Dose	Paw volume(ml) and % inhibition	
		90 min	180 min
I	Control	0.60±0.08	1.02±0.08
II	Inodomethacin 10mg/kg	0.25±0.02**	0.36±0.01**
III	MESP (200mg/kg)	0.51±0.05*	0.88±0.05*
IV	MESP(400mg/kg)	0.29±0.01**	0.42±0.08**

Statistical comparison: Each group (n=5), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed.$^{*}P<0.01,\ ^{**}P<0.001$

5.10.Analgesicactivity of *Sphenodesme paniculata*

Anti nociception is propertydefind as reduction in response of sensory nerve system to noxius stimuli.

*5.10.1.*Hot Plate Test

Effect of methanolic extract of Sphenodesme paniculatasignificantly showed protective effect in heat induced pain in hot plat method in mice.Methanolic extract 400 mg per oral showedmaximum analgesic effect in hot plat test in mice.morphine at dose 5 mg /kg significantly increased pain latency

Table.22: Antinociceptive activity of alcoholic extract by using Hot Plate Test

Groups	Treatment and Dose	Mean latency before and after drug administration			
		0 min	30 min	60 min	90 min
I	Control	2.30	2.50	2.44	2.32
II	Morphine 5mg/kg	1.96	4.56**	8.92**	11.53**
III	MESP (200mg/kg)	2.01	2.9*	3.86*	5.13*
IV	MESP(400mg/kg)	2.12	4.39**	6.72**	6.14***

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed.,$^{*}P<0.05$, $^{**}P<0.001$

5.10.2. Acetic acid induced writhing

Effect of methanolic extract of Sphenodesme paniculata significantly showed protective effect in chemical induced pain (acetic acid) method in mice. Methanolic extract 400 mg per oral showed maximum analgesic effect in acetic acid induced writhing in mice. Diclofenac at dose 50mg /kg significantly increased pain latency

Table22: analgesic activity of *Sphenodesme paniculata* by using acetic acid induced writhing

Groups	Treatment and Dose	No: of animals	Average number of writhing	Percentage inhibition
I	Control	6	62.60±1.72	_
II	Diclofenac 50mg/kg	6	30.80±2.65**	50.70
II	MESP (200mg/kg)	6	51.20±2.28*	18.21
IV	MESP(400mg/kg)	6	41.00±1.14**	34.50

Statistical comparison: Each group (n=6), each value represents Mean ± SEM. One way Annova followed by Dunnett's test was performed., *$P<0.05$, **$P<0.01$

SUMMARY AND CONCLUSIONS

I. Preparation of 95% methanolic extracts, ethyl acetate extracts, of *sphenodesme paniculata* stemby using Soxhlet extraction andthe aqueous extraction was carried out by boiling the coarsely powdered leaves with waterbased on the polarity

II. All the extracts were subjected to various phytochemical screening and the study reveals the presence of alkaloids, phenols, flavanoids, carbohydrate ,steroids, saponins and glycosides were found in the stem extracts of *sphenodesme paniculata*

III. Total phenol content (TPC) for the extracts of *sphenodesme paniculata* were determined by Folin-Ciocalteu's method and it was found that the methanolic extract contains high amount of total phenols which was about 68.6±0.02 mgGAE/g for the MESPand 41.4±0.02 mg GAE/g for the EESP.

IV. Total flavanoids content of all the extracts of *sphenodesme paniculata* were determined by aluminium chloride colorimetric assay. It was found found that the extracts contains high amount of total flavanoids which are about 28.47±0.01 mg QE/g for the MESP and 21.1±0.02 mg QE/g for the EESP.

V. Total antioxidant activity of theMESP extracts was performed.Total antioxidant capacity of MESP was found to be 342.8 µg/ml calculated as Ascorbic acid equivalent.

VI. In the present study, Simultaneously determinationof quercetin, rutin and gallic acid in *sphenodesme paniculata*stem is carried out by HPTLC in solvent system of toluene : ethyl acetate: formic acid :methanol (3:0.8:0.2).

VII. The MESP extracts were evaluated for its antioxidant potential. DPPH radical scavenging ability of the extracts was studied. The IC50 values were found to be 6.4 µg/ml ,extract showed potent anti oxidant activity

VIII. ABTS radical scavenging ability of the MESP extracts were studied. The IC50 values were found to be 5.97 µg/ml, extract showed potent anti oxidant activity

IX. The cytotoxicity of all the stem extracts of *sphenodesme paniculata* were performed by MTT assay using HeLacell lines.The cytotoxicity experiment showed that all tested extracts were non-cytotoxic to HeLa cells up to a concentration of 200 µg/ml

X. Antimicrobial activity of aii the stem extractof *sphenodesme paniculata* were performed by sensitivity study, all the extracts showed antimicrobial activity ,methanolic extract showed higher zone of inhibition compare to other extracts

XI. The MESP extracts wereused for the pharmacological evaluvation of Anti-inflammatory activity at the doses of 200 and 400 mg/kg, methanolic extract showed significant reduction in paw oedema in carrageenan and histamine induced model when compared with control to treated group.

XII. The MESP extracts were used for the screening of thermal and chemical induced nociception (Hot plate and Writhing response) at the doses of 200 and 400 mg/kg, methanolic extract showed significantly reduced thermal and chemical induced nociception when compared with control to treated group.

The obtained results from overall study supports that the stem extracts of *sphenodesme paniculata* having potent antioxidant activity, andante-microbial activity,The cytotoxicity studies on HeLa cell line proved that the extracts were not having any cytotoxic effect. plantexract showing significant activity in analgesics and anti inflamatory. It is concluded that stem of this plant have medicinal important and beneficial for humans

Suggestion for further research:

This study can be extended by;

- Finding the active constituents present in the plant *Sphenodesme paniculata* by using GC-MS and NMR Spectroscopy.
- Identifying the mode of actions of the active constituents which are accountable for the potent anti-inflammatory and analgesic properties of the methanolic extract of the leaves of *Sphenodesme paniculata.*
- Evaluation for its therapeutic activity can be used for new drug discovery.

REFERENCES

1. Gurib-Fakim A, "Medicinal plants: traditions of yesterday and drugs of tomorrow," *Molecular Aspects of Medicine*, 2006; 1(27): 1–93
2. Aremu A.O,Amoo S.O, Ndhlala A.R, Finnie J.F, and Van Staden J, "Antioxidant activity, acetylcholinesterase inhibition*,Food ChemToxicol*. 2011;49(5):1122-8.
3. Aremu A.O, Amoo S.O, Ndhlala A.R, Finnie J.F, Van StadenJ.Iridoid content andmutagenic evaluation of *Leucosideasericea,"Food ChemToxicol*. 2011 ;49(5):1122-8.
4. Jayamathi T,Komalavally N, PandiyarajanV*,Asian plant sci*.Res,2012, 2 (6):688-691
5. EansW.C, Trease and Evans pharmacognosy, 4thedition. W.B.saunderscompanyLtd., london, 1996;593-597
6. KokateC.K, Purohit A.P,GokaleS.B,Pharmacognosy 7 th edition NiraliprakShan pune.,1997;105-144
7. Newman D.J, CraggG.M,Snader K.MNatural product as asource of new drugs over the period 1981-2002.*journel of natural product2003;66:1022-1027*
8. Newman D.J, CraggG.M,Snader K.M.The influence of natural products upon drug discovery.*natural product reports* 2000;17:215-217
9. Clardy j,walsh C.Lessons from natural molecules. *Natural product reports* 2004;829-832
10. Chevalliera A.*The encyclopedia of medicinal plants*, Dorling kindersly Ltd London 1996,10-13
11. Binu S, in his study " Medicinal plants used for treating body pain by the tribals in Pathanamthitta district, Kerala, india.*indian journal of traditional knowledge* 2011;10(3):547-549
12. RyanGB,MajnoG.Acuteinflammation.Americanjournal of pathology.1977;86(1):185-18
13. Harsh mohan text book pathology.6th edition chapter 6: inflammation and healing.2010;130-148
14. Mary J.pathophysiology.1st edition chapter 1; pathophysiolgy of inflammation 2008; 912.
15. Robbins and cotran .pathological basis of disease. 7ihe edition .Elsevier sunders chapter 2; acute and chronic inflammation 2005;47-69

16. Philadelphia P.A ,Baltimore,Kluwer W. A massage therapist guide to pathology,4 the edition,2009:59-61
17. ChandrasomaP,TaylorRC.Generalpathology,the host response to tissue injury,3 rdedition.McGraw-Hill ;2005:135-41
18. TharlotteS.E,Theanatomy and physiology of pain.*Basic science2009*;27(12);507
19. Helme R.D, et al,Neural Pathways in Chronic Pain, *Medicaljournal Australia;*1990(153)400-406
20. Shipton E.A, Pain, Acute & Chronic. W.U.P.1993 (ISBN 1-86814-241-8).
21. Wall P.D, Melzack R, Textbook of Pain, Fourth edition. Orlando, Harcourt Publishers; 1999:107-41
22. Lynn T, SchaibleK.S ,Textbook of Peripheral and Central Mechanisms of Pain Generation 2006: 177:3–28
23. Vanderah TW, Ossipov MH, Lai J, Porreca F. Cholecystokinin(CCK) as an endogenous mediator of opioidinduced paradoxicalpain and antinociceptive tolerance. In: Smith HS,ed. Drugs for Pain. Philadelphia;2012:255-262.
24. jennifer HE, Claudia BP,physiology and treatment of pain in critical care nurse2008;28(6):30-40
25. marchents.the physiology of pain mechanism:fromperipher to the brain.2008;34:285-286
26. Byers M, Bonica JJ. Loeser JD, Butler SH, Chapman CR, et a Peripheral pain mechanisms and nociceptor plasticity. Management of Pain. 3rd ed: Lippincott Williams & Wilkins; 2001:26-72.
27. medicinal plant used for treating body pain by the tribals in pathanamthitta district keralaindia.indian journal of traditional knowledge 2011;10(3):547-549
28. Saneja A, Kaushik D, Khokra SL, KaushikP,Sharma C and Aneja K.R, Evaluation of activities of Mitragynaparvifolia fruit extract, Journal of Natural Products, 2, 2009, 49-54.
29. Charulata N, Gulecha V, Chavan N and Deshmukh T, Formulation and evaluation of Analgesic and Antiinflammatory activity of Nytcanthesarbour-tritisGel,Indian Drugs, 47(8) August 2010, 66-68.

30. . Sathisha A, Udupa L, RaathanakarUP,Pai PG, Acharya SD and Shastry R, Anti-inflammatory and Analgesic activity of Phyllanthusniruri in Rodent models. Indian Drugs, 46(12) December, 2009,50-53.
31. ShaahbadkarGS, SwamyP, Gaikwad DR and Muttagi S, Antiinflammatory, Analgesic and Antimicrobial effects of SterciliafoetidaL.seed cake, Indian Drugs, 47(1) January, 2010, 52-56.
32. Solanki R, Madat D, Chauhan K, Adeshara SP, Analgesic Activity of Holarrhenaantidysenterica (Apoynaceae) Bark International Journal of Pharmacognosy and Phytochemical Research, 2(4), 2010, 5-7.
33. 5. V.VinothPrabhu, G.Nalini, N.Chidambaranathan, S. SudarshanKisan, Evaluation Of Anti Inflammatory And Analgesic Activity Of TridaxProcumbens Linn Against Formalin, Acetic Acid And Cfa Induced Pain, International Journal Of Pharmacy And Pharmaceutical Sciences, 3(2), 2011, 126-130.
34. HarishaCR,AshokBK,AcharyaR,SuklaVJ,Ravishankar B., Anti Inflammatory And Analgesic Activity Of Roots And Stem Of CissusRepedaVahl.Pharmacognosy Journal, 21(18), 2010,7-54.
35. Vittalrao AM, Shanbhag T, Kumari KM, Bairy KL And Shenoy S, Evaluation of Antiinflammatory and Analgesic Activitiesof Alcoholic Extract Of KaempferiaGalanga In Rats, Indian J PhysiolPharmacol, 55 (1),2011, 13–24
36. Bukhari IA, Khan RA, Gilani AU, Shah AJ, Hussain J, Ahmad VU, The analgesic, anti-inflammatory and calcium antagonist potential of Tanacetumartemisioides. Arch Pharm Res. 30(3), 2007 Mar,303-12.
37. Joseph JM,SowndhararajanK,ManianS,Evaluation of Analgesic and Anti-inflammatory Potential of Heydyotisparberula(G.Don)R.Br.ex.Arn. in experimental animal models.FoodChemToxicol, 48(7), 2010,July ,1876-80.
38. Silva J, Abebe W, Sousa SM, Duarte VG, Machadoand MIL, Matos MJA,Analgesic and anti-inflammatory effects of essential oils of Eucalyptus , Journal of Ethnopharmacology, 89(2-3), 2003, 277-283.
39. Bettina M.Ruppett,Edna F.R. Peveria,Lilia C. Gonccalves and Nuno A. Pereira. Pharmacological screening of plants recommended by Folk medicine as anti-snake

venom-1, analgesic and Anti-inflammtoryactivities.,Mem.Inst. Oswaldo Cruz, Rio de Janeiro, 86(II), 1991,203-205

40. . Jayarama Reddy, Gnanasekaran D, Vijay D. and Ranganathan TV, In vitro studies on anti asthmatic, analgesic and anti convulsant activities of the medicinal plant Bryonialaciniosa.Linn, International Journal of Drug Discovery, 2(2), 2010, 01-10
41. R.Vijayamirtharaj, S.Vincent and N.Senthilkumar, Analgesic activity of clerodendrumphlomidislinn. (Aerial parts), International Journal of Research in Pharmaceutical and Biomedical Sciences, 2 (1), 2011, 120-123.
42. Nanda BK, Jena J, Rath B, Behera BR, Analgesic and Antipyretic activity of whole parts of Sphaeranthusindicus Linn., Journal of Chemical and Pharmaceutical Research, 1 (1),2009,207-212 49. Patel A, Patel T,
43. Patel A, Patel T, Macwan C, Patel M, Chauhan K, Patel J, Evaluation of Anti inflammatory and Analgesic activity of roots of Rubiacordifolia in rats. J. Pharm. Sci. & Res. 2 (12), 2010, 809-813.
44. AnnamalaiPandurangan, RatanLalKhosa and Siva Hemalatha, Evaluation of Anti-inflammatory and Analgesic Activity of Root Extract of SolanumTrilobatum Linn, Iranian Journal of Pharmaceutical Research, 7 (3),2008, 217-221.
45. Islam MR,Mannan MA, Kabir MHB, Islam A and Olival KJ, Analgesic, anti-inflammatory and antimicrobial effects of ethanol extracts of mango leaves, J. Bangladesh Agril. Univ. 8(2), 2010, 239–244.
46. Kumar S, Kumar V, Deepa K, ChandrashekharMs, Evaluation of Analgesic And Anti-Inflammatory Activity of AsystasiaDalzelliana Extract, Journal Of Herbal Medicine And Toxicology, 5 (1),2011, 23-26 .
47. Owoyele BV, Olaleye SB, Oke JM, Elegbe RA, Anti - Inflammatory and Analgesic Activities Of Nothospondiasstaudtinigerian, Journal Of Physiological Sciences 19(1-2), 2004,102-105.
48. Apu AS, Liza MS, Jamaluddin ATM, Amran MD, Saha K, Nasrin N. The phytochemical screening and *In Vitro* bioactivities of the extracts of aerial part of *Boerhaviadiffusa* Linn. *Asian Pac J Trop Med*. 2012; 2(9): 673-678.

49. Velioglu, YS, Mazza, G, Gao, L, Oomah, BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J. Agric. Food .Chem*. 1998; 46: 4113-4117.
50. FZhishen J, Mengcheng T, Jianming W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*. 1999; 64: 555-559.
51. Ashok BT, Ali R. The aging paradox: Free radical theory of aging. ExpGerontol. 1999; 34: 293–303.
52. Aruoma OI. Methodological consideration for characterization for potential antioxidant actions of bioactive components in plants foods. *Mutat Res*. 2003; 532: 9–20.
53. Attarde DL, Kadu SS, Chaudhari BJ, Kale SS, Bhamber RS. *In vitro* antioxidant activity of pericarp of *Cucurbita maxima*Duch. ex Lam. *Int J Pharm Tech Res.*2010; 2(2): 1533-1538
54. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cationdecolorization assay. *Free Radic.* 1999; *Biol. Med*. 26, 1231–1237
55. Raghu KI, Ramesh CK, Srinivasa TR, Jamuna KS. Total antioxidant capacity in aqueous extracts of some common vegetables. *Asian J. Exp Biol. Sci.* 2011; 2(1): 58-62.
56. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Imm Meth*. 1983; 65, 55-63.
57. Antibacterial activities of the methanol extracts of Albiziaadianthifolia, Alchornealaxiflora ,Laporteaovalifolia and three other Cameroonian plants against multi-drug resistant Gram-negative bacteriaSaudi Journal of Biological Sciences 2016;21: 143-152.
58. P.D. Diwanand, Y.A.Gadhikar .Assessment Of Phytochemical Composition And Antibacterial Activity Of Different Extracts Of *BarleriaPrionitis*Leaves Against Oral Microflora To Improve Dental Hygiene. Asian Journal Of Pharmaceutical And Clinical Research, 2012; 5(2): 182-184

59. Kamal RaiAneja, Radhika Joshi, Chetan Sharma. Potency of *Barleriaprionitis*L. bark extracts against oral diseases causing strains of bacteria and fungi of clinical origin. New York Science Journal 2010; 3(11): 5-11
60. Patel A, Patel T, Macwan C, Patel M, Chauhan K, Patel J, Evaluation of Anti inflammatory and Analgesic activity of roots of Rubiacordifolia in rats. J. Pharm. Sci. & Res. 2 (12), 2010, 809-813.
61. AnnamalaiPandurangan, RatanLalKhosa and Siva Hemalatha, Evaluation of Anti-inflammatory and Analgesic Activity of Root Extract of SolanumTrilobatum Linn, Iranian Journal of Pharmaceutical Research, 7 (3),2008, 217-221.
62. Islam MR,Mannan MA, Kabir MHB, Islam A and Olival KJ, Analgesic, anti-inflammatory and antimicrobial effects of ethanol extracts of mango leaves, J. Bangladesh Agril. Univ. 8(2), 2010, 239–244.
63. Suchita Mittal, Praveen .K. Dixit. In-vivo anti-inflammatory and anti-arthritic activity of ethanolic extract of Asparagus Racemosus roots. International Research Journal of Pharmacy, 2013; 4(4): 167-172
64. Solanki R, Madat D, Chauhan K, Adeshara SP, Analgesic Activity of Holarrhenaantidysenterica (Apoynaceae) Bark International Journal of Pharmacognosy and Phytochemical Research, 2(4), 2010, 5-7.
65. Shenawy SM, Abdel-Salam OM, Baiuomy AR, Baeran SE, Arbid MS. Studies on the Anti-inflammatory and Anti-nociceptive effects of melatonin in rat. Pharmacol Res 2002;46:235-243
66. Bentley GA, Newton SH, Starr J. Studies on the anti-nociceptive action of drugs and their interaction with opioid mechanism against. Br J Pharmacol 1983;79:125-134
67. . Koster R, Anderson M, De Beer EJ. Acetic acid for analgesic screening. Fed Proc 1959; 18:412-415.
68. Vinegar R, Schreiber W, Hugo RJ. Biphasic development of carrageenan edema in rats. J PharmacolExpTher 1969;166:96-103